Universidade de Trás-os-Montes e Alto Douro

Genetic diversity and phylogenetic relationships in cowpea revealed by chloroplast DNA analysis

Dissertação de Mestrado em Genética Molecular Comparativa e Tecnológica

Eliana Maria Ribeiro Monteiro

Orientador: Professor Doutor Valdemar Pedrosa Carnide

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VILA REAL, 2017

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Resumo

O feijão-frade (*Vigna unguiculata* L. Walp) pertence à família Fabaceae e é originário de África. É uma leguminosa de grão de grande valor nutritivo, capaz de tolerar diferentes stresses, tais como secura, temperaturas elevadas e stresses do solo, tais como, a baixa fertilidade, solos ácidos, básicos e pouco drenados. O estudo da diversidade genética é uma área de pesquisa importante porque só uma avaliação tão completa quanto possível desta variabilidade permite a utilização do germoplasma no melhoramento de plantas. A diversidade genética pode ser avaliada através de carateres morfológicos e de marcadores moleculares. Existe um crescente interesse no uso do ADN cloroplastidial em estudos de populações, uma vez que a conservação dos genes neste genoma permite desenhar primers universais, o que facilita estudos filogenéticos de populações de indivíduos relativamente afastados.

Neste trabalho, uma colecção de *landraces* de feijão-frade do Sul da Europa foi caraterizada por parâmetros morfológicos e agronómicos. A diversidade e relações genéticas existentes nestas *landraces* comparativamente com acessos de outras partes do mundo, e com outras espécies de *Vigna* foram estudadas também através de marcadores microssatélites cloroplastidiais (cpSSRs).

Ao nível das caraterísticas qualitativas avaliadas nas *landraces* do Sul da Europa, os hábitos de crescimento ereto e semi-ereto foram os mais frequentes (44% e 42%, respetivamente); a folha terminal de forma sub-sagitada foi o tipo preponderante (44%); as duas cores de flor observadas foram branca (72%) e roxa (28%); e as sementes tinham, maioritariamente, cor creme (94%) com hilo preto (58%) e forma de rim (69%). Relativamente às caraterísticas quantitativas verificou-se ser o peso total das sementes a caraterística com maior coeficiente de variação (62,54 %) e o tamanho da vagem a caraterística com menor coeficiente de variação (15,29 %). O peso de 100 sementes apresentou um elevado valor de heritabilidade (h^2 = 0,98). A análise de agrupamentos, efetuada através do método Ward com base em 10 caraterísticas morfo-agronómicas, repartiu as *landraces* desta coleção por três grupos distintos, não se tendo observado nenhuma relação entre as *landraces* em cada um dos grupos e a sua origem geográfica. A análise em componentes principais (PCA) mostrou que os primeiros três componentes principais explicam 82,1 % da variação total.

Um conjunto de 10 pares de *primers* foi utilizado para analisar a diversidade genética de 108 acessos de *Vigna unguiculata* compreendendo as subespécies *alba*, *pubescens*, *tenuis* e *unguiculata* (var. *spontanea* e var. *unguiculata,* cultigrupo *unguiculata* e cultigrupo *sesquipedalis*) e ainda 5 acessos de outras espécies de *Vigna* (*V. racemosa, V. radiata* e *V. mungo*), incluindo maioritariamente acessos cultivados mas também silvestres. Oito dos 10 *loci* (ccmp3, ccmp7, VgcpSSR1, VgcpSSR10, VgcpSSR12, VgcpSSR14, ccSSR4, cSSR7) revelaram-se polimórficos ao nível das várias espécies estudadas. O conjunto dos 34 diferentes alelos detetados combinaram-se em 10 haplótipos diferentes, oito dos quais únicos. O haplótipo mais frequente (90.3%), putativamente ancestral, incluiu acessos cultivados de *V. unguiculata* ssp. *unguiculata* cultigrupo unguiculata e *V. unguiculata* ssp. *unguiculata* cultigrupo sesquipedalis, e acessos silvestres de *V. unguiculata* ssp. *unguiculata* var. *spontanea* e *V. unguiculata* ssp. *tenuis*.

O presente estudo permitiu mostrar a grande diversidade ainda existente no feijão-frade em Portugal e outros países do Sul da Europa, apesar do baixo polimorfismo detetado no seu genoma cloroplastidial. Verificou-se ainda a existência de haplótipos partilhados por material cultivado e silvestre. A grande variabilidade detetada na coleção de feijão-frade agora estudada e a partilha de haplótipos revela-se de grande importância para programas de melhoramento desta espécie.

Palavras-chave: *Vigna unguiculata* L. Walp; *landraces*; caraterísticas morfológicas e agronómicas; marcadores moleculares; cpSSRs

Abstract

Cowpea (*Vigna unguiculata* L. Walp) belongs to the family Fabaceae and is native to Africa. It is a nutritious grain legume, able to tolerate different stresses, such as drought, high temperatures and tolerates most soil stresses, such as low fertility, acidic, basic and poorly drained soils. The study of genetic diversity is an important research area because only an evaluation as complete as possible of this variability allows the use of germplasm in plant breeding. Genetic diversity can be evaluated using morphological traits and molecular markers. There is a growing interest in the use of cloroplastidial DNA in studies of populations, because with the conservation of the gene in this genome, allows to design universal primers, which facilitates phylogenetic studies of populations of relatively remote individuals.

In this study, a collection of cowpea landraces from Southern Europe was characterized by morphological and agronomic traits. The diversity and genetic relationships in this landraces comparatively with accessions from other parts of the world, and with other species of *Vigna*, were also studied through chloroplast microsatellite markers (cpSSRs).

At the level of the qualitative traits evaluated in the landraces of Southern Europe, the erect and semi-erect growth habits were the most frequent (44% and 42%, respectively); sub-hastate shape (44%) was the most occurring terminal leaflet type; the two flower colours observed were white (72%) and purple (28%); and seeds had, mostly, cream colour (94%) with black hilum (58%) and kidney shape (69%). In relation to the quantitative characteristics, was verified that the total seed weight was the characteristic with the highest coefficient of variation (62.54%) and the pod length with the lowest coefficient of variation (15.29%). The 100 seeds weight presented a high value of heritability ($h^2 = 0.98$). The cluster analysis performed using the Ward method based on 10 morphological and agronomic characteristics, divided the landraces of this collection in three distinct groups, and was not observed relationships between the landraces in each group and their geographical origin. Principal component analysis (PCA) showed that the first three major components accounted for 82.1% of the total variance.

A set of 10 pairs of primers were used to analyse the genetic diversity of 108 accessions of *Vigna unguiculata* including *alba*, *pubescens*, *tenuis* and *unguiculata* subspecies

(var. *spontanea* and var. *unguiculata*, cultigroup unguiculata and cultigroup sesquipedalis) and 5 accessions of other species of *Vigna* (*V*. *racemosa*, *V*. *radiata* and *V*. *mungo*), including mostly cultivated, but also wild. Eight of the 10 loci (ccmp3, ccmp7, VgcpSSR1, VgcpSSR10, VgcpSSR12, VgcpSSR14, ccSSR4, cSSR7) were polymorphic at the level of the various species studied. The set of 34 different detected alleles were combined into 10 different haplotypes, eight of which were unique. The most frequent haplotype (90.3%), putatively ancestral, included cultivated accessions of *V*. *unguiculata* ssp. *unguiculata* cultigroup unguiculata and *V*. *unguiculata* ssp. *unguiculata* cultigroup sesquipedalis, and wild species of *V*. *unguiculata* ssp. *unguiculata* var. *spontanea* and *V*. *unguiculata* ssp. *tenuis*.

The present study allowed to show the great diversity still existing in cowpea in Portugal and other Southern Europe countries, despite the low polymorphism detected in its chloroplastidial genome. It was verified the existence of haplotypes shared by cultivated and wild material. The great variability detected in this collection of cowpea studied and the sharing of haplotypes is of great importance for breeding programs of this species.

Key-words: *Vigna unguiculata* L. Walp; landraces; morphological and agronomic traits; molecular markers; cpSSRs

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List of abbreviations, acronyms and units of measurement

°C – Degrees Celsius

% – Percentage

µl – Microliters

µM – Micromolar

AFLPs - Amplified Fragment Length Polymorphisms

bC – Before Christ

BSA – Bovine serum albumin

bp – Base pair

cm – Centimeter

cpDNA – Chloroplast deoxyribonucleic acid

cpSSR – Chloroplast Simple Sequence Repeats

CV – Coefficient of variation

DNA – Deoxyribonucleic acid

dNTPs – Deoxyribonucleotide triphosphates

FC - Flower colour

g – Grams

GH - Growth habit

g/kg – Grams/kilogram

***h* -** Genetic diversity

h^2 – Heritability

ha – Hectare

HC - Hilum colour

***I* -** Shannon's information index

IBPGR – International Broad for Plant Genetic Resources

IR – Inverted repeat

ISSR – Inter Simple Sequence Repeats

IITA – International Institute of Tropical Agriculture

kb – Kilobase

KCl – Potassium chloride

kg/ha – kilograms/hectare

K_2O – Potassium oxide

LSC – Large Single-Copy

mm – Millimeters

mM – Millimolar

Mbp – Mega base pair

mg - Milligrams

MgCl2 – Magnesium chloride

mg/kg – Milligrams/kilogram

Mha – Million hectares

MT - Metric tonnes

***Ne* -** Number of effective alleles

ng/µl – Nanogram/microliter

nSSRs – Nuclear Simple Sequence Repeats

NPK – Nitrogen, phosphorus and potassium

NSP - Number of seeds per pod

NTSYSpc – Numerical Taxonomy and Multivariate Analysis System for personal computer

PC 1 – Principal Component 1

PC 2 – Principal Component 2

PC 3 – Principal Component 3

PCA - Principal Component Analysis

PCR - Polymerase Chain Reaction

pH – Potential of hydrogen

PL - Pod length

P_2O_5 – Phosphorus oxide

r - Number of replicates

RAPDs - Random Amplified Polymorphic DNA

RFLPs – Restriction Fragment Length Polymorphisms

SC - Seed colour

SD – Standard deviation

s_e^2 - Residual variance

s_g^2 - Genetic variance

SNPs - Single Nucleotide Polymorphisms

SPSS –Statistical Package for the Social Sciences

SS - Seed shape

SSC – Small Single-Copy

SSRs - Simple Sequence Repeats

SW - 100 seeds weight

TLS - Terminal leaflet shape

T_{max} – Maximum air temperature

T_{min} – Minimum air temperature

ton/ha – Tonnes/hectare

TSW - Total seed weight

U – Units

UPGMA – Unweighted Pair Group Method with Arithmetic Mean

USA – United States of America

UTAD – University of Trás-os-Montes and Alto Douro

W/m2 – Watt/square meter

w/v – Weight/volume

1. Introduction

1.1. *Vigna unguiculata* L. (Walp.)

1.1.1. General considerations

Cowpea (*Vigna unguiculata* L. Walp) belongs to the family *Fabaceae* and is native from Africa (Singh et al., 1997; Kotze, 2015). This grain legume is a diploid species (2 n = 2 x = 22) and its genome size has been estimated at 620 Mbp (Arumuganathan and Earle, 1991;Timko and Singh, 2008). Cowpea has been identified since antiquity by Dioscorides, and was described by Linné in 1760 from a cultivated form collected from the Caribbean, known as *Dolichos unguiculatus* (Badiane et al., 2014).

Cowpea is one of the most adapted, versatile and nutritious grain legumes, since it is able to tolerate different stresses compared with other crop species, such as drought, high temperatures and most soil stresses, grows well in most types of soil, from heavy clays, when well drained, to sandy soils, however, prefers sandy loam or sandy soils, which tends to be less restrictive on root growth (Ehlers and Hall, 1997; Pan et al., 2014). It grows in a wide range of pH, but responds better in slightly acidic to slightly alkaline soils (pH 5.5-8.3) (Ehlers and Hall, 1997). Like other legume crops, cowpea has the ability to grow in low fertile soils due to its capacity to fix atmospheric nitrogen through symbiosis with nodule bacteria (*Bradyrhizobium* spp.) (Kalloo and Bergh, 1993; Singh et al., 1997). Through this feature, about 40-80 kg nitrogen/ha go back into the soil, while the total amount of nitrogen fixation is about 70-350 kg/ha, so cowpea growing rotation with cereal crops can help to restore soil fertility (Kalloo and Bergh, 1993; Singh et al., 1997; Badiane et al., 2012; Tan et al., 2012; Kotze, 2015). The roots of cowpea also form a symbiotic association with mycorrhiza, which improves soil's available phosphorous content (Valenzuela and Smith, 2002). Moreover, contributes for the incorporation of organic matter in the soil which improves its structure and fertility, water infiltration and soil water holding capacity (Valenzuela and Smith, 2002). Cowpea has a great efficiency in the use of the soils, it prevents and controls soil erosion due to being shade tolerant, a quick grower and a rapid ground covering species (Singh et al., 2003). These attributes make cowpea an important crop component of subsistence farmers around the world (Kotze, 2015). However, it is susceptible to a variety of pests and diseases, which includes, fungal, bacterial and viral diseases, insect pests, nematodes and parasitic plants (Singh and Allen, 1979).

Cowpea, “the crop of all-round utilization”, is grown for dry seeds, immature seed, immature green pod, green leaves, and even roots (Kalloo and Bergh, 1993). It is an important warm-season legume, growing in tropical and subtropical regions (Timko and Singh, 2008). The most important part of the plant for human consumption are the dry seeds, which are used in a variety of dishes according to the countries/regions cultural traditions (Timko and Singh, 2008). In many parts of Africa particularly during the “hungry period”, between August and September, cowpea hay is also critical in the feeding of animals (Tan et al., 2012). Cowpea is also important for medical use and is often used in the treatment of various diseases. The roots are used in the treatment of diseases such as epilepsy and chest pain and the seeds to treat amenorrhea (Van Wyk and Gericke, 2000; Zia-ul-haq et al., 2010).

As other grain legumes, cowpea is an important source of minerals and vitamins (folic acid and vitamin B), present in the young leaves, seeds and pod (Singh et al., 1997; Timko et al., 2007). Amino acids profile reveals that lysine, leucine and phenylalanine contents are relatively high, although methionine, cysteine and tryptophan are low (Kalloo and Bergh, 1993). Cowpea is called “poor man’s meat”, because the protein contents range from about 26% – 28% in green leaf, and to 23% – 32% in seeds (Table 1) (Iqbal et al., 2006; Tan et al., 2012). It provides cheap major source of protein for millions of people throughout the developing world, which complements diets based on cereal grains or starchy food (Timko and Singh, 2008). When compared to cereal and tuber crops, cowpea has a lower fat content and its protein content is about two to four fold higher which made it and excellent crop (Timko and Singh, 2008; Kotze, 2015).

Table 1 - Chemical composition of cowpea seeds and leaves. (Adapted from Gómez (2004); Iqbal et al (2006); Tan et al. (2012)).

	Seeds (%)	**Leaves (%)**
Carbohydrate	55-56	8
Protein	23-32	26-28
Water	11	85
Crude fibre	5,9-7,3	2
Fat	4,8	0,3
Phosphorous	0,146	0,063
Calcium	0,076-0,104	0,256

1.1.2. Taxonomy

Grain legumes are one of the most well-known botanical families and have a great economic importance. They are part of a set of species belonging to the family *Fabaceae*, formed by more than 19,000 species (Silva et al., 2009). The genus *Vigna* contains more than 80 agricultural important species, being divided into several subgenera based on morphological characteristics, extent of genetic hybridization/reproductive isolation and geographical distribution of the species (Vijaykumar et al., 2009; Badiane et al., 2014; Kotze, 2015). The various subgenera include the African subgenera *Vigna* and *Haydonia*, the Asian subgenus *Ceratotropis*, and the American subgenera *Sigmoidotropis* and *Lasiosporon* (Vijaykumar et al., 2009). *Vigna* genus comprises agriculturally important species such as mungbean (*Vigna radiata* L. Wilczek) and black gram (*Vigna mungo* L. Hepper), cultivated species of the Asian subgenus *Ceratotropis*. *Vigna radiata*, is one of the important pulse crops of India and has easily digestible protein, while *Vigna mungo* is an important summer pulse crop of many South Asian countries (Ghafoor et al., 2001; Makeen et al., 2007).

Cowpea belongs to the group of dicotyledons, the order *Fabales*, family *Fabaceae*, subfamily *Faboideae*, tribe *Phaseoleae*, subtribe *Phaseolinae*, genus *Vigna*, species *unguiculata* (Kalloo and Bergh, 1993; Singh et al., 1997). The classification and nomenclature of cowpea and its associated subspecies and varieties is somewhat unclear due different author's classification. *Vigna unguiculata* L. Walp has 11 subspecies that differ from one another with respect to various morphological characteristics (Vijaykumar et al., 2012). Five of the subspecies, ssp. *baoulensis*, ssp. *burundiensis*, ssp. *letouzeyi*, ssp. *aduensis*, and ssp. *pawekiae*, are perennial, allogamous, adapted to humid environments and are mainly recognized by their floral characteristics. Five other subspecies, ssp. *dekindtiana* (var. *spontanea*), ssp. *stenophylla*, ssp. *tenuis*, ssp. *alba*, and ssp. *pubescens*, are wild, perennial, autogamous and are recognized by their vegetative traits showing their adaptation to drier and coastal environments (Singh et al., 1997; Vijaykumar et al., 2009; Vijaykumar et al., 2012; Badiane et al., 2014). Only one subspecies is annual (ssp. *unguiculata*), comprising wild (var. *spontanea*) and cultivated (var. *unguiculata*) forms (Vijaykumar et al., 2012). The var. *spontanea* is the progenitor of cultivated cowpea and is a savannah taxon that often grows as a weed in and around cultivated fields (Vijaykumar et al., 2012). All cultivated cowpea are grouped under *V. unguiculata* spp. *unguiculata* and are sub-divided into four cultigroups, namely: unguiculata, which is the common form and

grown as a pulse; biflora, which is characterized by small erect pods and used as a forage; sesquipedalis, commonly known as "Yard-long Beans", characterized by its very long pods which is mostly produced in Asia; and textilis, a primitive cultivar, which was used for fibres obtained from its long floral peduncles (Ehlers and Hall, 1997; Coulibaly et al., 2002). Pasquet (1998) also proposed the insertion of melanophthalmus (black-eyed pea) as another cultigroup.

1.1.3. Origin and domestication

All the evidence points to the origin of cultivated cowpea in Africa, although the exact location of its domestication is still uncertain. Among African regions, Ethiopia, Central Africa, South Africa and West Africa are considered the probable domestication centres (Ba et al., 2004; Huynh et al., 2013). This grain legume is one of the earliest sources of human food and has probably been used as a crop plant since Neolithic times (Kalloo and Bergh, 1993). In the African context, the role of cowpea is predominantly as a pulse. The cultivated cowpea, *Vigna unguiculata* ssp *unguiculata* var. *unguiculata* belongs to the unguiculata cultigroup. After the introduction of unguiculata forms in India and Southeast Asia, two other cultigroups have evolved, sesquipedalis and biflora, under a predominant influence of human selection (Kalloo and Bergh, 1993). The cultigroup unguiculata is the largest and includes most of the African grains. Members of the biflora cultigroup are common in India, while textilis cultigroup is a fairly rare and has been used in Africa as a source of fibre. Sesquipedalis has apparently evolved in Asia and is rare in African germplasm (Timko et al., 2007). The propagation of cowpea in Asia occurred in the third millennium bC. It was introduced in Europe around 300 bC, where it remains as a smaller crop in the Southern part. The crop was introduced to the tropical Americas between the 16th and 17th centuries from Africa by the Spanish in the course of the slave trade (Singh et al., 1997).

Some authors claim that *Vigna unguiculata* ssp *dekindtiana* var. *mensensis* was the progenitor of the modern cowpea (Kalloo and Bergh, 1993; Ehlers and Hall, 1997), however, the wild *Vigna unguiculata* ssp. *dekindtiana* var. *spontanea* (also referred as var. *dekindtiana*) is accepted as the most likely progenitor of domesticated cowpea. Its morphology and growth habit are very similar to those of cowpea landraces, although it also possesses wild-like attributes such as shattering pods with small seeds (Coulibaly et al., 2002; Fang et al., 2007).

The centre of diversity of wild *Vigna* species is Southeast Africa. The African origin of the cowpea has never been a point of contention, since wild forms only exist in Africa, including Madagascar island (Fang et al., 2007). Despite the wide distribution of var. *spontanea* throughout sub-Saharan Africa, molecular studies point to a unique domestication event (Coulibaly et al., 2002). However, there is disagreement about the geography of this domestication. There are two theories in which the areas of domestication vary between West and Northeast Africa.

The theory of the domestication centre of cowpea in West Africa was proposed by Ng and Maréchal (1985) and Vaillancourt and Weeden (1992). This theory is based on the high level of morphological diversity for cultivated cowpea; in the existence of wild hybrids resulting from the crossing between the wild and cultivated forms; on archaeological evidence of cowpea in Ghana; and also on the molecular similarities observed in the chloroplast DNA between wild species and cultivated forms of Nigeria (Coulibaly et al., 2002; Badiane et al., 2014). West Africa region (Nigeria, Niger, Burkina Faso, and Ghana) is a major centre of diversity of cultivated cowpea, based on morphological examinations of over 10,000 accessions (Vaillancourt and Weeden, 1992; Ehlers and Hall, 1997). Several studies have concluded that it was probably domesticated by farmers in this region. Others studies indicate that weedy cowpea, intermediate between cultivated and wild types, are common in West Africa, and proposed that cowpea was domesticated from weedy plants growing in Nigeria (Vaillancourt and Weeden, 1992; Coulibaly et al., 2002).

The theory of the domestication centre of cowpea in West Africa was contradicted by isoenzymatic and ethnobotanical studies, which revealed the absence of wild diversity in this region and a higher level of genetic diversity in accessions of Ethiopian origin (Coulibaly et al., 2002; Fang et al., 2007; Badiane et al., 2014).

The theory of Northeast Africa as domestication centre is based on studies of Baudoin and Maréchal (1985) that demonstrated the absence of varieties ecologically real savage in West Africa; in the great morphological diversity of the wild forms in the region that goes from Ethiopia to South Africa; and in results of ethnobotanical, linguistic and isoenzymatic studies, conducted by Pasquet and Fotso (1994). There is also evidence that domestication of cowpea in Northeaster Africa could have occurred simultaneously with the domestication of sorghum (*Sorghum bicolor*) and pearl millet (*Pennisetum typhoides*) in the third millennium bC (Coulibaly et al., 2002).

In order to understand the gene pool of African cowpeas, to determine its relationship with the African wild and non-African cultivated cowpea and to clarify the origin and dispersion of this culture, a recent study using a worldwide collection of cowpea, including varieties from Africa, Asia, Europe, North and South America and a collection of African wild ancestral cowpea (*V. unguiculata* ssp. *dekindtiana*), and Single Nucleotide Polymorphisms (SNPs), was developed by Huynh et al. (2013). This study revealed the existence of two genetic pools: one gene pool in Western Africa and a largest one in Eastern Africa (Figure 1). Those authors also verified that West and East African *dekindtiana* formed two distinct groups suggesting a divergent domestication process, as it happens in common bean (*Phaseolus vulgaris* L.).

Figure 1 - Worldwide distribution of gene pools of cowpea landraces. Gene pool 1 is represented in blue and gene pool 2 represented in red. Relative proportions of blue and red colours for each symbol represent the likelihood of an accession assigned to gene pools 1 and 2, respectively. (Adapted from Huynh et al. (2013)).

1.1.4. Cultivation and production

Cowpea is able to maintain some growth or at least survive under drought conditions partly due to its deep rooting habit. It grows well under 400 mm to 700 mm of rainfall but is often grown with less than 400 mm of rainfall (Kotze, 2015). As most the grain legumes crops, cowpea cannot withstand waterlogged conditions (Valenzuela and Smith, 2002). Is often cultivated in hot low elevation equatorial and subtropical areas of the world, usually below 1300 m above sea level (Ehlers and Hall, 1997). Grows in a wide range of temperature throughout all stages of development but with an optimum of 28 °C for reproductive development (Singh et al., 1997). Cowpea is often intercropped with

several cereal, root crops, cotton, sugarcane because it is shade-tolerant (Ehlers and Hall, 1997; Singh et al., 2003).

In 2013, the worldwide production of cowpea dry seeds was about 8.2 metric tonnes on about 12.1 million hectares. Sub-Saharan African countries are the main producers and account for 97% of worldwide production (Table 2) (FAOSTAT, 2017). In Europe the production is quite low, accounting for only about 0.3% of world production and in this continent cowpea is mainly cultivated in four countries: Serbia, Former Yugoslav Republic of Macedonia, Croatia and Bosnia and Herzegovina (Table 2). Cowpea is also produced in the Asian and American continents, however the production is very low, being the contribution of these countries to the world production of 1.8% and 1%, respectively (Table 2) (FAOSTAT, 2017). In Europe there is a deficit production of all food grain legumes, especially beans, lentils, chickpeas and cowpea, being peas an exception despite their advantageous characteristics (Schneider, 2002).

Since 2013, India is the world's largest importer of grain legumes, and Canada is the largest exporter (FAOSTAT, 2017). From the geographical point of view, the production of grain legumes is very concentrated. India is the main producer of pulses, producing around a quarter of the 4.4 million tonnes world production in 2013 (FAOSTAT, 2017), probably because its population is mostly vegetarian being grain legumes an important source of protein (Schneider, 2002). However, grain legume per capita consumption has shown a slight decline over time in both developed and developing countries, declining from 7.6 kg/person/year in 1970 to 6.1 kg/person/year in 2006 (FAOSTAT, 2017). Middle East / North Africa is the only region where the per capita consumption of this type of legumes increased from 6.2 kg/person/year to 7.1 kg/person/year during the same period (FAOSTAT, 2017). In many parts of Europe, since 1980, there has been a decline in the cultivation of grain legumes (Rochon et al., 2004; Voisin et al., 2013).

Globally, international trade in grain legumes has grown faster than its production. It is likely that in the future, international trade in grain legumes will continue to grow, as it meets many demands of today’s society (Schneider, 2002). Many developing countries will continue to rely on imports to meet their grain legume consumption needs, as the constraints associated with grain legume production may not be easily solved. As a result, current production of grain legumes may not be enough to respond to demand (FAOSTAT, 2017).

Table 2 - Largest cowpea producers per continent in 2013.(Adapted from FAOSTAT (2017)).

Continent	Country	Production (MT)	Area (Mha)	Yield (MT/Mha)
Africa	Nigeria	4630.54	3593.3	1.29
	Niger	1794.89	5130.9	0.35
	Burkina Faso	569.39	1200.5	0.47
	United Republic of Tanzania	188.72	234.2	0.81
	Mali	168.27	254.4	0.66
Asia	Myanmar	115.10	134.4	0.86
	China, Mainland	15.20	12.5	1.22
	Sri Lanka	14.19	10.8	1.31
	Philippines	1.20	0.3	4.00
	Iraq	1.05	0.2	5.25
America	Haiti	30.50	42.0	0.73
	United States of America	28.99	15.7	1.85
	Peru	18.82	15.5	1.21
	Trinidad and Tobago	0.50	0.2	2.50
	Jamaica	0.28	0.3	0.93
Europe	Serbia	15.82	4.5	3.52
	Former Yugoslav Republic of Macedonia	7.60	2.0	3.80
	Croatia	0.32	0.7	0.46
	Bosnia and Herzegovina	0.30	0.2	1.50

1.2. Cowpea diversity characterization

Cowpea is described as an annual, warm season herbaceous plant with a variety of growth habits that can be either erect, semi-erect, prostate or climbing (Badiane et al., 2014) (Figure 2). Emergence of cowpea seedling after germination is epigeal, where the cotyledons emerges from the ground first during germination (Kotze, 2015). The full life cycle from germination to dry grain production requires from 60 days to more than 150 days depending on the genotype (Badiane et al., 2014). Leaf shape can be globose, sub–globose, hastate and sub–hastate and the flower colour varies between white, mauve-pink, purple and yellow (IBPGR, 1982) (Figure 3). The crop is autogamous but around 5 % outcrossing was reported in the cultivated varieties, probably due to insect activities (Badiane et al., 2014; Kotze, 2015). Usually two or three pods per peduncle are common,

but if the growing conditions are favourable, four or more pods can be present. Pods are cylindrical and can be either curved or straight with eight to twenty seeds per pod. Seeds can be smooth or wrinkled, with cream, white, brown, black, buff or red colour. Seed shape ranges between kidney, globose, ovoid, rhomboid and crowder (IBPGR, 1982 ; Badiane et al., 2014; Kotze, 2015).

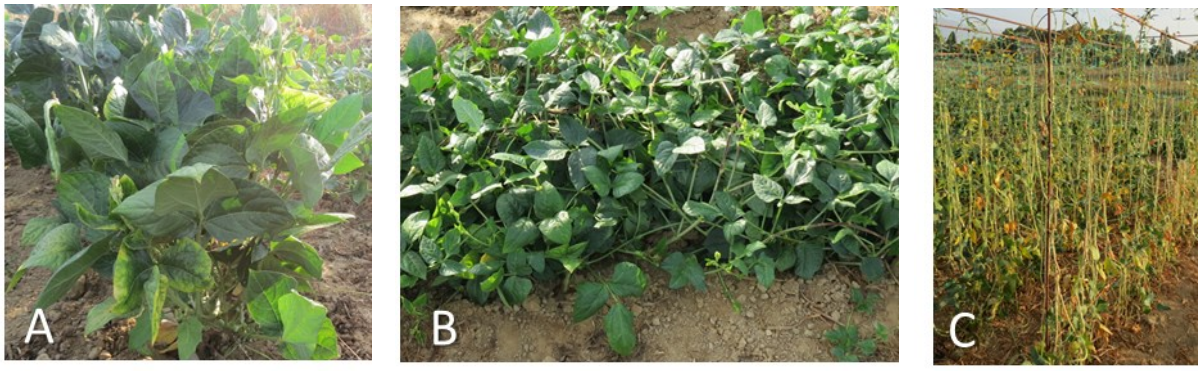

Figure 2 - Growth habits in cultivated cowpea. Erect (A), prostrate (B) and climbing (C). (Photographed by author).

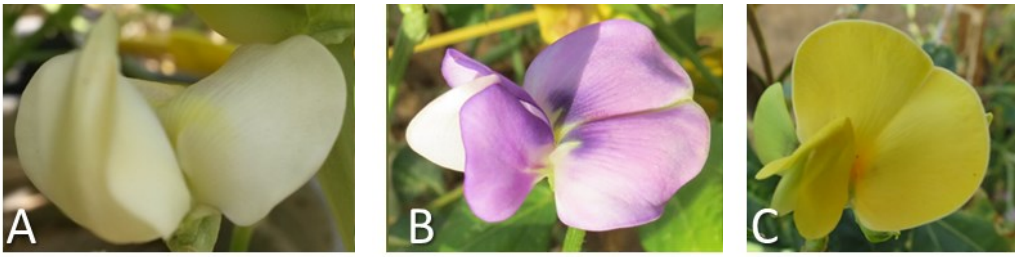

Figure 3 - Colours of cowpea flowers. White (A), purple (B) and yellow (C). (Photographed by author).

Cowpea has been cultivated worldwide with more incidence in tropical areas and displays a high phenotypic/morphological variability (Pandey and Dhanasekar, 2004; Xavier et al., 2005; Timko et al., 2007). Genetic diversity is an important research area because the accurate assessment of genetic variability is useful for the preservation and utilization of germplasm resources and improvement of varieties/cultivars (Tan et al., 2012).

Traditional crop varieties, generally referred as "landraces", but also known as "farmer varieties", "local varieties" or "primitive varieties", were continually maintained by farmers because of their culinary preferences, cultural and socio-economic context

(Veteläinen et al., 2009; Stoilova and Pereira, 2013). According to Zeven (1998), it is impossible to define precisely what is a landrace. At the second meeting of the On-Farm Conservation and Management Taskforce of the European Cooperative Group on Genetic Resources (Bioversity International) held in Stagerlitz (Germany) in 2006 the following definition was proposed: "A landrace of a seed-propagated crop is a variable population, which is identifiable and usually has a local name. It lacks 'formal' crop improvement, is characterized by a specific adaptation to the environmental conditions of the area of cultivation (tolerant to the biotic and abiotic stresses of that area) and is closely associated with the uses, knowledge, habits, dialects, and celebrations of the people who developed and continue to grow it."

It is thought that the wild progenitors gave rise to the earliest primitive varieties or primitive forms. Initially, these primitive varieties must have been genetically quite narrow, however, other populations of wild progenitor may have subsequently been domesticated, and the genetic flow between wild relative and cultivated species crops may have extended the genetic base over time, resulting in diverse landraces (Veteläinen et al., 2009). Wild relatives of crops are adapted to several environments, maintaining a high genetic diversity, which contrasts with the loss of genetic diversity during domestication and human selection of cultivated material (Li et al., 2001). This way, desirable traits such as biotic and abiotic stress resistances and special nutritional values, important for crop improvement, can be found in some wild germplasm of grain legumes (Pasquet, 1999).

Germplasm characterization, namely landraces, can be done using phenotypic traits and molecular markers. Genetic markers have been used since the beginning of plant breeding not only as an indirect selection mean of the advantageous or disadvantageous characteristics of the species, but are also very useful in taxonomic studies, phylogenetic and genetic analyses (Godwin et al., 1997). The genetic diversity within and between groups of plant species is routinely performed using various techniques such as morphological, biochemical and molecular markers.

1.2.1. Morphological and agronomic characterization

Several studies report the characterization of cowpea by morphological and agronomic traits (Pasquet, 1998; Adewale et al., 2011; Stoilova and Pereira, 2013; Cardona-Ayala et al., 2013). This characterization is made using a set of parameters: i) related with the plant morphology, such as growth habit, terminal leaflet shape, flower colour, days to flowering and to mature pods, seed shape and colour; and ii) related with plant production, namely number of pods per peduncle and per plant, number of seeds per plant, 100 seeds weight and seed weight.

Morphological and agronomic characterization does not require any complex equipment or experiments, being simple and inexpensive to score. These reasons are responsible for the constant use of morphological and agronomic traits as the first step in studies of characterization and genetic relationships (van Beuningen and Busch, 1997; Magloire, 2005). The main disadvantage of this type of characterization is that the observed characteristics do not reflect exclusively the genotype, but reflect the environment and the effects of the interaction genotype and environment (Magloire, 2005).

1.2.2. Molecular characterization

In the 70's of the last century began a research field called "molecular biology", one of the most memorable genetic developments and that, even today, continues to have a revolutionary impact on biology. Technologies such as DNA molecular markers are being increasingly used in breeding programs, in order to increase the selection efficiency and germplasm characterization and maximizing genetic gain, allowing access and selection of variability at the level of DNA (Guimarães, 2005). DNA molecular markers are genetic markers based on individual nucleotide sequence variation, which are the direct reflection of genetic polymorphisms at the DNA level (Staub et al., 1996). An ideal molecular marker should be: i) polymorphic; ii) multiallelic; iii) codominant, that means no intra-locus interaction and a heterozygous hybrid simultaneously presents the traits of the homozygous parents; in a progeny, the heterozygotes can be distinguished from each of the homozygotes; iv) non-epistatic, its genotype can be inferred from its phenotype, whatever the genotype at other loci may be, meaning no inter-locus interaction; v) "neutral", the allelic substitution at the marker locus do not have phenotypic or selective effects; almost

all molecular polymorphisms are neutral; vi) insensitive to environment, the genotype can be inferred from the phenotype, no matter what the environment is (Vienne, 2003).

Different types of molecular markers have been used in phylogenetic studies, molecular systematics, evolutionary biology, characterization of plant genetic resources, genetic diversity estimation and germplasm management (Staub et al., 1996; Ferreira et al., 2015).

1.2.2.1. Nuclear markers

In recent years, with the rapid development of molecular biology, molecular markers based on PCR have been widely used in genetic studies of various crops, such as cowpea, namely RAPD, AFLPs, SSR and SNP markers.

RAPDs (Random Amplified Polymorphic DNAs) where widely used in cowpea genetic analysis because the technique is simple and requires small amounts of DNA. Ba et al. (2004) evaluated the genetic variation and the relationships between 26 landraces and 30 wild cowpea species from West, Eastern and Southern Africa and concluded that wild accessions were more diverse in East Africa, which is the likely area of origin of *V. unguiculata* var. *spontanea*. Malviya et al. (2012) analysed genetic diversity among 10 Indian cultivars of cowpea with 18 sets of RAPD markers and verified a variation in genetic diversity among these cultivars ranging from 0.1742 to 0.4054.

Coulibaly et al. (2002) used AFLPs (Amplified Fragment Length Polymorphisms) to assess the genetic diversity and evaluate genetic relationships in a total of 117 *Vigna* accessions, including 47 domesticated cowpea (ssp. *unguiculata* var. *unguiculata*), 52 wild and weedy annuals (ssp. *unguiculata* var. *spontanea*), and 18 perennial accessions of the wild subspecies *pubescens*, *tenuis* and *alba*. They concluded that wild annual cowpea (var. *spontanea*) was more diverse than domesticated cowpea, and wild cowpea in Eastern Africa was more diverse than in Western Africa, suggesting an Eastern African origin for the wild taxon. Fang et al. (2007) examined with these markers the genetic relationships among 60 advanced breeding lines from six breeding programs in West Africa and USA, and 27 landrace accessions from Africa, Asia, and South America. Principal coordinates analysis show a clustering of breeding lines by program origin, indicated a lack of genetic diversity compared to potential diversity.

nSSRs (nuclear Simple Sequence Repeats), also known as microsatellites, are specific regions of DNA sequence that contain clusters of tandem repeats motifs of length

1-6 nucleotides (Kapil et al., 2014). Some studies demonstrate that the nuclear Simple Sequence Repeats (nSSRs) can detect more polymorphisms than RFLPs (Restriction Fragment Length Polymorphisms), RAPDs, and AFLPs in grain legumes, being the most frequently used marker in the genetic diversity analysis of cowpea (Badiane et al., 2012; Kapil et al., 2014). Li et al. (2001) used 46 nuclear microsatellite DNA markers to evaluate genetic similarities among 90 cowpea breeding lines developed at IITA (International Institute of Tropical Agriculture). A total of 27 primer pairs could amplify polymorphic single-locus microsatellites from all lines. They verified that, by means of only five polymorphic microsatellite primers, 88 of the 90 cowpea lines could be distinguished. Badiane et al. (2012) developed a set of 44 nSSRs polymorphic primer combinations for cowpea and evaluated the genetic diversity and phylogenetic relationships among 22 local cowpea varieties and inbred lines collected throughout Senegal, finding that with few exceptions the local varieties clustered in the same group and the inbred lines were in a second cluster.

SNPs (Single Nucleotide Polymorphisms) are powerful tools in genetic diversity study in living organisms and are more effective in diversity assessment compared with other markers. A worldwide collection of cowpea with total of 422 landraces and a total of 46 African ancestral wild cowpea, was genotyped with more than 1,200 SNP markers revealing the presence of two major gene pools in cultivated cowpea in Africa (Huynh et al., 2013). In 2014 Egbadzor et al., characterized 113 cowpea accessions comprising of 108 from Ghana and five from abroad using 458 polymorphic SNP markers. The authors concluded that SNP markers were more efficient in discriminating among the cowpea germplasm than morphological, seed protein polymorphism and simple sequence repeat studies, reported earlier on the same collection.

1.2.2.2. Chloroplast Simple Sequence Repeats (cpSSR) markers

Chloroplasts are organelles that contain their own genome and are considered to be derived from the endosymbiosis of cyanobacteria (Kapil et al., 2014). This organelle is present in plants and algae, and besides the photosynthesis, is also involved in various metabolic pathways essential for the plant, such as amino acid biosynthesis, pigments and vitamins (Mota and Aragão, 2005; Kapil et al., 2014).

The chloroplast genome is highly conserved in size, structure, gene content and linear order of genes among related species and even between phylogenetically distant

species, because the chloroplastidial DNA (cpDNA) does not undergo recombination and has a low rate mutation (Palmer, 1985; Vaillancourt and Weeden, 1992; Kapil et al., 2014). The arrangement of the circular chloroplast genome is extremely conserved with genes generally occurring in the same order (Palmer, 1985). As the rate of evolution of cpDNA is slow, in terms of base substitution as well as structural rearrangements, it makes this molecule interesting to study patterns of genetic differentiation between populations and between regions. With few exceptions, circular chloroplast genome typically possess a large single-copy (LSC) and a small single-copy (SSC) region separated by two large inverted repeat (IR) sequences (Figure 4) (Maréchal-Drouard et al., 1991; Castro et al., 2013; Chen et al., 2016). The LSC region is slightly less conserved than the rest of the chloroplast genome, and hence potentially more useful for studies at low molecular levels (Grivet et al., 2001). Among angiosperms, the chloroplast genome varies little in size, structure, and gene content. The typical chloroplast genome in angiosperms ranges in size from 135 to 160 kb and is characterized by a large inverted repeat (Olmstead and Palmer, 1994). *Vigna unguiculata* chloroplast genome is 152,415 bp in length (Figure 4) (Mota and Aragão, 2005). There is a growing interest in the use of cpDNA in genetic studies of plant populations, because the conservation of the gene arrays in cpDNA it is possible to design several "consensual" or "universal" chloroplast primers, which facilitates phylogenetic and genetic studies of populations (Demesure et al., 1995). Due to its haplotype nature and usually maternal inheritance the cpDNA is important in taxonomy, population genetics, systematic studies, species distribution and population differentiation (Palmer, 1985; Vaillancourt and Weeden, 1992; Kapil et al., 2014).

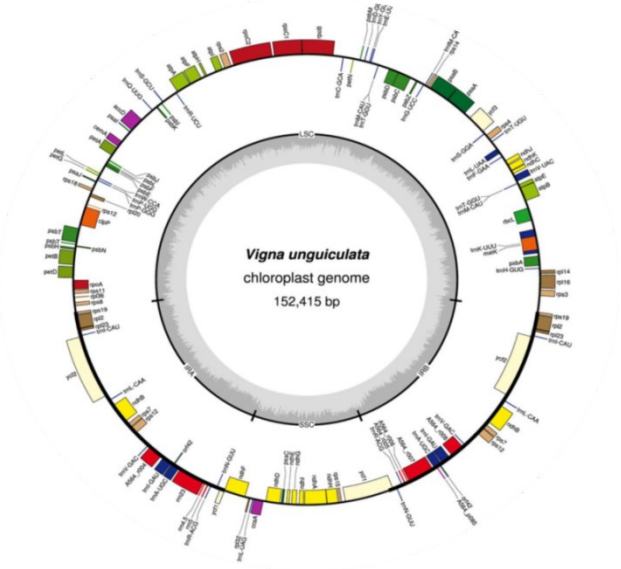

Figure 4 - Representation of regions of chloroplast genome in cowpea. LSC - large single-copy; SSC -small single-copy; IRa and IRb - inverted repeat sequences.(Adapted from Mota and Aragão (2005)).

The cpDNA polymorphism is a powerful tool for understanding patterns differentiation between plant populations, depending on the evolution rate and the inheritance standard. These properties make this marker interesting for studying patterns of genetics among populations and between regions, and has been used to infer historical events such as possible recolonization routes (Avise, 1994). The information on genetic variation within and between populations added to the information obtained by cpDNA allows inferring about the geographic distribution and historical variation at the genetic level, and can be used to determine species diversity centres and therefore it is very useful in decision making on the choice of populations or priority conservation areas (Crandall et al., 2000).

Chloroplast simple sequence repeats or chloroplast microsatellites (cpSSRs) were developed for genetic analyses in the 1990s (Chung and Staub, 2003). This technology is based on highly polymorphic regions and has been used for the genetic analyses of the chloroplast genome of several species such as *Glycine* (Powell et al., 1996), *Hordeum* (Provan et al., 1999), *Oryza* (Provan et al., 1996) and *Pinus* (Powell et al., 1995).The cpSSR markers can be used to detect DNA variability in the chloroplast genome and has the same characteristics as nuclear microsatellites. Moreover, cpSSR markers are found to be polymorphic and transferable among related species because the flanking regions of cpSSR loci are highly conserved (i.e., low nucleotide substitution rates when compared to the nuclear genome) (Chung and Staub, 2003). This way, an important advantage of cpSSR is that no knowledge of the target species genome sequence is required for analyses when making use of universal primers. Unlike what happens with the nuclear dominant markers, which have limitations due to their biparental inheritance, the uniparental inherited cpSSRs, allow overcoming this limitation and complement their information (Ferreira et al., 2015).

1.3. Objectives

- To characterize a collection of landraces from Southern Europe through morphological and agronomic traits;
- To evaluate the genetic diversity in a worldwide cowpea collection by chloroplast microsatellite markers;
- To study the genetic relationships among different*Vigna*species using chloroplast microsatellite markers.

2. Material and methods

2.1. Morphological and agronomic characterization of cowpea landraces from Southern Europe

2.1.1. Plant material and experimental design

One trial was installed at University of Trás-os-Montes and Alto Douro (UTAD), Vila Real, Portugal (N 41°17'51", W 07°44'12", 465 m), with 36 landraces from four Southern Europe countries, being 12 from Portugal, 12 from Spain, 8 from Italy and 4 from Greece (Table 3). Sowing was in the first week of June. From each landraces 10 seeds were hand sowed in one row with 2 m length, with a distance between rows of 0.75 m and between seeds of 0.20 m. The topsoil (0-20 cm) was classified as gleyic fluvisol with a medium texture and presented 1.61 g/kg humus content, 44 mg/kg of P_2O_5, 110 mg/kg of K_2O_2 and a pH (KCl) 5.2. Before sowing the experimental field was ploughed with a rotary tiller and fertilized with 250 kg/ha of nitromagnesium 27 and 200 kg/ha of NPK (Ca-Mg-S) 8-12-12 (2-2-14). The trail was drip irrigated from beginning July until end of August.

The average maximum (T_{max}) and minimum (T_{min}) air temperature (°C) and total rainfall (mm) per month (from May to September) were recorded at weather stations located in the experiment location (Table 4).

2.1.2. Morphological and agronomical traits

The set of 36 landraces were phenotyped by six qualitative characters (growth habit, terminal leaflet shape, flower colour, seed shape, seed coat and hilum colour) and four quantitative characters (pod length, number of seeds per pod, 100 seed weight and total seed weight) based on IBPGR descriptors (IBPGR, 1982). For the quantitative characters five random pods were analysed and for the parameter 100 seed weight, two random samples of the total seed produced by each accession were weighted.

Table 3 - Landraces number, subspecies, country of origin and status of the accessions of V. unguiculata ssp. unguiculata (L.) Walp characterized.

Accession number	Bank code[#]	Country	Locality	Status	Common Name
Vg11	Vg11	Portugal	Torre de Moncorvo	Landrace	Feijão frade
Vg13	Vg13	Portugal	Alijó	Landrace	Feijão frade
Vg18	Vg18	Portugal	Mirandela	Landrace	Feijão frade
Vg47	Vg47	Portugal	Almeida	Landrace	Feijão frade
Vg52	Vg52	Portugal	Trancoso	Landrace	Feijão frade
Vg59	Vg59	Portugal	Fundão	Landrace	Feijão frade
Vg94	CP5553	Portugal	Sertã	Landrace	Feijão frade
Vg95	CP5556	Portugal	Mértola	Landrace	Feijão frade
Vg97	CP5648	Portugal	Abrantes	Landrace	Feijão frade
Vg99	CP5651	Portugal	Ponte de Sor	Landrace	Feijão frade
Vg104	CP5554	Portugal	Sousel	Landrace	Feijão frade
Vg252	Vg252	Portugal	Baião	Landrace	Feijão frade
Vg212	BGE002195	Spain	Orense	Landrace	Carilla
Vg217	BGE019751	Spain	Gerona	Landrace	Frijol d'hiver
Vg220	BGE022147	Spain	Granada	Landrace	Friguelo
Vg222	BGE024703	Spain	Baleares	Landrace	Fesol
Vg223	BGE025201	Spain	Caceres	Landrace	Carilla
Vg232	BGE047731	Spain	Pontevedra	Landrace	Cajabicho
Vg239	BGE036461	Spain	Huelva	Landrace	Carilla
Vg241	BGE039236	Spain	Jaen	Landrace	Jiguelo
Vg244	BGE035390	Spain	Badajoz	Landrace	Frailiño careto
Vg245	BGE028976	Spain	Albacete	Landrace	Ciriguello
Vg248	BGE040426	Spain	Zamora	Landrace	Carilla
Vg249	BGE039237	Spain	Cordoba	Landrace	Higuelo
Vg161	AUA1	Greece	-	-	-
Vg162	AUA2	Greece	-	-	-
Vg208	MG 106823	Greece	-	Landrace	Mavromatica
Vg209	MG 107571	Greece	Creta	Landrace	Lianofasula
Vg185	4354	Italy	-	-	-
Vg187	5426	Italy	Cuneo	-	-
Vg193	MG 115107	Italy	Abruzzo	-	-
Vg196	MG 113767	Italy	Basilicata	Cultivated form	Fagiolo dall'occhio torto
Vg197	MG 115525	Italy	Puglia	-	-
Vg200	MG 113832	Italy	Campania	Cultivated form	Fagiolini nani
Vg204	MG 113779	Italy	Puglia	Cultivated form	Fagiolini pinti baresi
Vg206	MG 112248	Italy	Sicilia	-	-

[#] Vg, University of Trás-os-Montes and Alto Douro, Vila Real, Portugal; CP, National Institute for Agricultural and Veterinary Research (INIAV), Elvas, Portugal; BGE, National Plant Genetic Resources Centre-National Institute for Agricultural and Food Technology Research (CRF-INIA), Alcalá de Henares, Spain; AUA, University of Athens, Athens, Greece; MG, Institute of Biosciences and Bioresources (IBBR), Italian National Research Council (CNR), Bari, Italy.

Table 4 - Average of solar radiation precipitation, mean, minimum and maximum temperature and relative humidity during May to September of 2016 and for the period 1981-2010.

Year/ Period	Month	Solar radiation Dgt [W/m2]	Precipitation [mm]	Air temperature [°C]			Relative humidity [%]
				Mean	Min.	Max.	
2016	**May**	200.19	124.4	14.2	8.5	21.1	74.3
1981-2010		X	70.7	14.9	9.4	20.4	N.A.
2016	**June**	282.05	25.2	19.1	11.1	27.7	67.9
1981-2010		X	33.7	19.2	12.8	25.5	N.A.
2016	**July**	306.76	0.2	23.7	14.3	33.6	51.4
1981-2010		X	15.1	21.3	14.3	25.5	N.A.
2016	**August**	252.08	0.2	23.3	14.0	33.4	47.9
1981-2010		X	26.5	21.7	14.8	28.6	N.A.
2016	**September**	198.90	28.4	19.6	11.4	30.7	61.1
1981-2010		X	54.8	18.5	12.6	24.4	N.A.

2.1.3. Statistical analysis

The qualitative traits frequencies were determined manually. Minimum, maximum and mean values, standard deviations, coefficients of variation, F value and heritability were calculated for the quantitative traits. The heritability of each quantitative trait was calculated using the following equation: $h^2 = (s_g^2) / [s_g^2 + (s_e^2/r)]$, where s_g^2 and s_e^2 represent the genetic and residual variance for each trait and r the number of replicates of each landrace (Gitonga et al., 2014). The treatment of quantitative data and the calculation of significant differences through the Tukey test were performed using the summary statistics procedure in SPSS program version 8.0. The principal component analysis (PCA) and construction of the dendrogram by Ward method, based in the 10 morphological traits (growth habit, terminal leaflet shape, flower colour, seed shape and colour, hilum colour, pod length, number of seeds per pod, 100 seed weight and seed weight per plant) were performed using the Past3 program.

2.2. Chloroplast SSR analysis of *Vigna unguiculata* and other *Vigna* species

2.2.1. Plant material and DNA extraction

A total of 113 accessions, 66 of which from Iberian Peninsula, were analysed (Tables S1, S2 and S3). These accessions comprehend *Vigna unguiculata* ssp. *unguiculata* wild (var. *spontanea*) and cultivated (var. *unguiculata*) forms of both unguiculata and sesquipedalis cultigroups; wild forms of other *V. unguiculata* subspecies, ssp. *alba*, ssp. *pubescens* and ssp. *tenuis* and also other *Vigna* species, namely *V. racemosa, V. mungo* and *V. radiata*.

For each accession, young and healthy leaves, with about 4 cm, were collected and stored at -80ºC until use. For DNA extraction, the leaf tissues were disrupted using the TissueLyser equipment (Qiagen, Chatsworth, USA) and DNA purified using the plant DNA extraction kit NucleoSpin Plant II (Macherey-Nagel, Düren, Germany), following manufacturer instructions. The extracted DNA was checked by electrophoresis on 1.0% agarose gels, quantified on the spectrophotometer Nanodrop ND-1000 (Thermo Fisher Scientific, Rockford, USA) and diluted to 10 ng/µL in water.

2.2.2. Chloroplast SSR-PCR amplification

Ten pairs of primers were used to amplify the cpSSR *loci* and the forward primer of each pair was fluorescently labelled (Table 5). The amplifications were carried out separately for each cpSSR locus, in a thermal cycler (Biometra, Göttingen, Germany) and PCR conditions were optimized based in protocols of Weising and Gardner (1999), Chung and Staub (2003) and Lei Pan et al. (2014). After amplification, two mixtures were made: the first mix included the primers: ccmp7, ccSSR4, ccmp3, VgcpSSR10, and ccSSR22; the second mixture included: ccmp10, VgcpSSR1, ccSSR7, VgcpSSR12 and VgcpSSR14.

The amplifications were performed in a final volume of 20 µl. For ccmp primers reaction mixture containing: 1 × *Taq* buffer, 0.025 mg BSA, 10 ng of genomic DNA, 2 mM MgCl2, 0.15 µM dNTPs, 0.4 µM for each primer, and 0.175 U *Taq* polymerase (NzyTech Lisboa, Portugal). For ccSSR primers and VgcpSSR primers reaction mixture contains: 1 × *Taq* buffer, 0.025 mg BSA, 10 ng of genomic DNA, 2.5 mM MgCl2, 0.2 µM dNTPs, 0.5 µ M for each primer, and 0.05 U *Taq* polymerase (NzyTech, Lisboa, Portugal).

Table 5 - Sequence, labelling, position in the cowpea genome, size and annealing temperature of amplification of the 10 cpSSR primer pairs used.

Locus	Repeat motif	Primer Sequence	Dye	Position in cowpea #	Location/ region#	Expected Size (bp)#	Annealing Temperature (^{0}C)
VgcpSSR1[a]	(TA) 5	F: GGTGGATGTTTATACCCAATCG R: TCTTTCTGCGATACAAACAAGAA	NED	7256-7277 7481-7503	*trnK-rbcL* IGS LSC	248	55
VgcpSSR10[a]	(AT) 5	F: GGGCTCATTGGCTGTAGAAA R: CCATCTCTCCCCAATTGAAA	PET	55732-55751 55876-55895	*trnR-trnS* IGS LSC	164	56
VgcpSSR12[a]	(AT) 6	F: GGCCATTTATCCCACTTTCC R: CCAGTCTCTACTGGGGGTTA	PET	64456-64475 64686-64705	*psbJ-psbL-psbF* IGS LSC	250	56
VgcpSSR14[a]	(AT) 5	F: TGGATCATAATCCTTGAACATCA R:TGCGAAAACAAAGATAAGAAATCA	VIC	113630-113652 113814-113836	*PsaC-ndhE* IGS SSC	208	59
ccSSR4[b]	(T)8	F: AGGTTCAAATCCTATTGGACGCA R:TTTTGAAAGAAGCTATTCARGAAC	VIC	54258-54236 53997-54020	*TrnR-AtpA* LSC	≈262	50
ccSSR7[b]	(T)11	F: CGGGAAGGGCTCGKGCAG R: GTTCGAATCCCTCTCTCTCCTTTT	FAM	28742-28725 28438-28461	*PsbC-TrnS* LSC	≈205	50
ccSSR22[b]	(T)8	F:CCGACCTAGGATAATAAGCYCATG R: GGAAGGTGCGGCTGGATC	FAM	132219-132242 132381-132398	*TrnL-16SrRNA* LSC	180	53
ccmp3[c]	(T)11	F: CAGACCAAAAGCTGACATAG R: GTTTCATTCGGCTCCTTTAT	FAM	54775-54755 54693-54712	*trnG intron* LSC	≈83	50
ccmp7[c]	(A)13	F: CAACATATACCACTGTCAAG R: ACATCATTATTGTATACTCTTTC	NED	9938-9919 9789-9812	*atpB-rbcL* IGS LSC	≈150	50
ccmp10[c]	(T)14	F: TTTTTTTTTAGTGAACGTGTCA R: TTCGTCGDCGTAGTAAATAG	NED	150400-150379 150304-150323	*rpl2-rps19* LSC	≈97	50

[a] Pan et al. 2014, [b] Chung and Staub 2003, [c] Weising and Gardner 1999, [#]Position and expected size according to primers homology with the published V. *unguiculata* L. complete chloroplast genome sequence available in Genebank (accession NC 018051.1).

2.2.3. Electrophoresis

To confirm amplification, 5 µL of loading dye (0.1% bromophenol blue, 0.1% xylene cyanol FF, 10% Ficol) were added to 7.5 µL of PCR product and the mixture submitted to electrophoresis in 2.5 % agarose gels (w/v), containing syber safe (NBS Biologicals, Cambridgeshire, UK) run for 1 h at a constant voltage of 150 V. The stained gels were imaged with a digital camera, and recorded using the Molecular Image® Gel-Doc™ XRþ with Image Lab™ Software (BIO RAD, Hercules, CA, USA). Dilutions of the PCR products were run on the ABI Prism® 3730 Genetic Analyzer using the GeneScan™500 LIZ® size standard (PE Applied Biosystems, Foster City, CA, USA).

2.2.4. Data analysis

Labelled products of cpSSRs were analysed and sized by means of Peak Scanner™ v1.0 free software (PE Applied Bio- systems, Foster City, CA, USA). Data analysis of cpSSR amplicons was performed by means of GenAlEx 6.5 software to determine allele frequency, number of effective alleles (*Ne*) using the following equation*:* $Ne = 1/\sum pi^2$; Shannon's information index (*I*) was calculated using equation: $I = -\sum pi \ log_2 \ pi$; and genetic diversity (*h*) calculated using the equation: $h = 1 - \sum pi^2$ (Peakall and Smouse, 2012). Haplotypic frequencies were calculated and a median-joining network analysis performed using the software NETWORK 5.0.0.1 (Fluxus Technology Ltd., Suffolk, England). Based on the same data, a dendrogram was constructed by UPGMA method with the software NTsys-pc, version 2.20 software package (Rohlf, 2005).

3. Results and discussion

3.1. Morphological and agronomic characterization of cowpea landraces from Southern Europe

Landraces or traditional old varieties have an important role in the introduction of improved adaptive characteristics (Stoilova and Pereira, 2013). These landraces are important in helping to deal with environmental and demographic changes, as they preserve the genetic diversity and help in breeding new crop varieties (Gixhari et al., 2014). So we must preserve the landraces because they are well adapted to the local environment, to disease resistance and, in addition, to prevent the erosion of plant genetic diversity, they promote their sustainable use (Veteläinen et al., 2009). It is known that selection acts to evolve the superior genotype and therefore genetic variability is a basic pre requisite for plant breeding program (Mishra et al., 2014). Despite the effectiveness of the use of molecular markers in diversity studies, morphological and agronomic traits remain imperative to plant breeders. As the grain-type cowpea cultigroup unguiculata is the most consumed in Europe, in this study the genetic structure and diversity is presented for one set of landraces from Southern European countries (Portugal, Spain, Italy and Greece).

During the period of morphological and agronomic characterization of this collection solar radiation, precipitation, temperature and relative humidity, were recorded during May to September of 2016 (Table 4). Solar radiation was higher in July 2016 (306.76 W/m2) and lower in September 2016 (198.90 W/m2). It was also verified that in 2016 the months of July and August registered the highest mean temperatures, 23.76°C and 23.31°C, respectively, such as in the long term period 1981-2010, with a mean temperature of 21.3°C and 21.7°C in July and August, respectively. As for precipitation, the year 2016 was significantly different comparing with the long term period 1981-2010. The most pronounced differences were observed in July and August. In the period 1981-2010, during these months were recorded 15.1 mm and 26.5 mm total precipitation, respectively and in the same months in 2016 were very dry and the precipitation extremely reduced, not exceeding 0.2 mm. Despite the scarce precipitation observed over the months of the characterization period, being cowpea a drought tolerant crop, eventually the production was not affected.

In this study was verified a great variability for the traits analysed in the 36 Southern Europe landraces. Qualitative traits are, in general, independent from environmental factors and are governed by one or few major genes (Govindaraj et al., 2015). Different types of growth habits were verified in this set of landraces being the growth habit erect (44%) the most frequent (Table 6). The growth habit prostrate was only observed in Greek landraces (Table 6). Growth habit is an important trait when deciding the planting density. The climbers, prostate and erect cowpea types can be used in different planting systems such as sole crop or intercropping (Egbadzor et al., 2014a).

Sub-hastate shape (44%) was the most occurring terminal leaflet type (Table 6). Hastate and globose terminal leaflets are conspicuous, however they are less frequent than sub-hastate and sub-globose types.

Two different flower colours, white (72%) or purple (28%) were observed (Table 6). Several morphological traits in cowpea, such as, seed colour and eye pattern are linked and so is flower colour (Kehinde et al., 1997; Egbadzor et al., 2014b). Some authors refer that there is a pleiotropic effect between flower, pod and seed coat pigmentation in cowpea (Egbadzor et al., 2012). The linkage of flower colour to other traits can help in using it in indirect selection for important economic traits (Egbadzor et al., 2014a).

The most common seed were cream colour (94%), black hilum (58%) and kidney shape (69%) (Table 6). In addition, the cream seeds were mostly from Iberian Peninsula. Black and brown seeds were only observed in Italian and Greek accessions, respectively (Table 9) and grey hilum in accessions from Spain (Table 6). It is known that high grain yield and grain quality are the primary breeding objectives of all cowpea breeding programs (Egbadzor et al., 2014a). However, seed colour is one of the characteristics that consumers look for in cowpea and this preference has cultural dimension. In Portugal seeds with cream colour and black hilum are preferred by consumers. This national preference can be explained by the cooking water does not becoming so dark when compared to brown or black seeds, and also due to historical and cultural links (Carvalho et al., 2016).

Table 6 - Qualitative traits frequencies in 36 cowpea landraces by country.

Qualitative trait		**Frequency**				
		Portugal	**Spain**	**Italy**	**Greece**	**Total**
Growth habit	Erect	0.50	0.50	0.38	0.25	0.44
	Semi-erect	0.50	0.42	0.50	-	0.42
	Prostate	-	-	-	0.25	0.03
	Semi-prostate	-	0.08	0.12	0.50	0.11
Terminal leaflet shape	Hastate	0.50	-	-	0.50	0.22
	Sub-hastate	0.25	0.67	0.38	0.50	0.44
	Globose	0.25	0.08	0.12	-	0.14
	Sub-globose	-	0.25	0.50	-	0.20
Flower colour	White	0.83	0.75	0.75	0.25	0.72
	Purple	0.17	0.25	0.25	0.75	0.28
Seed colour	Cream	1	1	0.88	0.88	0.94
	Brown	-	-	-	0.12	0.03
	Black	-	-	0.12	-	0.03
Hilum colour	Black	0.58	0.67	0.75	-	0.58
	Brown	0.33	0.25	-	0.25	0.22
	Grey	-	0.08	-	-	0.03
	Green	-	-	0.13	-	0.03
	Eye absent	0.09	-	0.12	0.75	0.14
Seed shape	Kidney	0.92	0.92	0.38	-	0.69
	Globose	0.08	0.08	-	0.50	0.12
	Ovoid	-	-	0.62	0.50	0.19

In contrast to qualitative traits, the quantitative traits are influenced by environmental factors. For the four quantitative traits, the minimum, maximum values and the mean, the standard deviation, the coefficient of variation, the F value and the heritability were determined (Table 7). The parameters that presented the highest F value were the 100 seed weight (148.96) and the pod length (9.88). Total seed weight was the character with the highest coefficient of variation (62.54 %) and pod length the one with the lowest (15.29 %) (Table 7). Heritability is used to indicate the relative degree to which

a character is transmitted from parent to offspring (Omoigu et al., 2006). However, high heritability alone is not enough to make efficient selection in the advanced generations unless accompanied by substantial amount of genetic advance (Mishra et al., 2014). Classifying the heritability in the classes high (>0.75), moderate (0.60-0.75) and low (<0.60) the parameter 100 seeds weight presented a high heritability (h^2 = 0.98), the pod length a moderate heritability (h^2 = 0.64) and the number of seeds per pod a low heritability (h^2 = 0.36) (Table 7). Omoigu et al. (2006) and Egbadzor et al. (2013) also reported in cowpea a high heritability in 100 seeds weight parameter, h^2 =0.98 and h^2 =0.96, respectively. High heritability for 100 seeds weight (h^2 =0.91) was also verified in soybean (*Glycine max* (L) Merrill), in a study of Aditya et al. (2013). Several studies in cowpea demonstrate that pod length has moderate to high heritability. Apte et al. (1987) verified a heritability of h^2 =0.62, Patil and Baviskar (1987) observed a heritability of h^2 =0.70 and Thiyagarajan (1989) showed a heritability of h^2 =0.71 in this character. In the case of seeds per pod some authors also verified a low heritability, such as Patil and Baviskar (1987) that verified a heritability of h^2 =0.33 and Sreekumar et al. (1979) of h^2 =0.41.

Table 7 - Mean values obtained for each of 36 cowpea landraces in four quantitative traits with their respective mean, standard deviation (SD), coefficient of variation (CV), heritability (h^2), F value and Tukey's test (for a significance level of 0.05).

Accession number	Pod length (cm)	Number of seeds per pod	100 seed weight (g)	Total seed weight (g)
Vg11	18.5	9.6	26.75	87.30
Vg13	18.6	10.6	21.00	65.80
Vg18	20.6	12	26.35	101.50
Vg47	18.7	10.6	28.25	82.00
Vg52	18.2	12.0	22.35	83.80
Vg59	18.0	14.0	15.00	168.20
Vg94	19.8	13.2	21.95	120.00
Vg95	16.4	7.0	32.40	11.30
Vg97	17.5	10.8	19.50	178.50
Vg99	20.1	12.6	21.30	149.00
Vg104	19.9	10.8	19.75	38.10
Vg252	20.6	12.6	27.85	110.30
Vg212	18.6	11.0	22.90	116.00
Vg217	16.8	11.0	24.45	109.30
Vg220	20.4	10.4	26.25	78.60
Vg222	21.0	10.4	27.20	109.80
Vg223	18.3	13.0	24.05	139.50
Vg232	17.8	10.6	24.25	54.40
Vg239	19.2	12.0	23.15	107.00
Vg241	17.6	9.8	24.80	57.30
Vg244	17.4	10.2	24.20	39.80
Vg245	15.9	12.8	23.30	206.80
Vg248	16.7	10.4	24.40	105.20
Vg249	19.0	11.4	28.95	113.00
Vg161	15.8	12.2	17.05	65.50
Vg162	12.5	10.2	14.10	16.60
Vg208	11.4	10.2	16.85	287.00
Vg209	13.5	11.0	16.45	130.00
Vg185	17.8	12.8	14.90	140.30
Vg187	21.1	11.6	25.00	58.50
Vg193	16.2	10.4	16.45	105.50
Vg196	18.1	13.2	16.50	125.50
Vg197	16.1	7.6	27.40	38.10
Vg200	15.6	10.2	13.75	43.80
Vg204	16.0	9.8	19.50	88.30
Vg206	15.0	9.8	20.30	336.50
Average	17.63	11.05	22.18	107.44
SD	2.69	2.11	4.72	67.20
CV (%)	15.29	19.11	21.28	62.54
h^2	0.64	0.36	0.98	-
F	9.88	3.88	148.96	-
$Tukey^{0.05}$	2.81	2.92	1.12	-

The dendrogram (Figure 5), based on 10 morphological and agronomic traits, grouped the 36 landraces in three clusters:

i) Cluster I, with five landraces: the Spanish accessions Vg212 and Vg245 in one sub-cluster (I.1) and the Italian accession Vg 206 and the Greek accessions Vg 208 and Vg 209 in another sub-cluster (I.2);

ii) Cluster II, comprising 12 cowpea landraces separated in two sub-clusters: sub-cluster II.1 containing only the Greek landrace Vg 162 and the sub-clusters II.2 with 11 landraces from Iberian Peninsula and Italy;

iii) Cluster III, with 19 landraces distributed in three sub-clusters: sub-cluster III.1 with only one of the Spanish landrace (Vg 220); sub-cluster III.2 with landraces from the four countries (Portugal, Spain, Italy and Greece) and sub-cluster III.3 containing landraces from Iberian Peninsula and Italy.

There's no evident relation between the geographic origin of the landraces and their clustering based on the morphological and agronomic traits analysed in the 36 Southern Europe landraces studied. This can reflect trades of cowpea material within Southern Europe countries, particularly in Iberian Peninsula, and also a common origin of the cowpea material cultivated in this part of Europe.

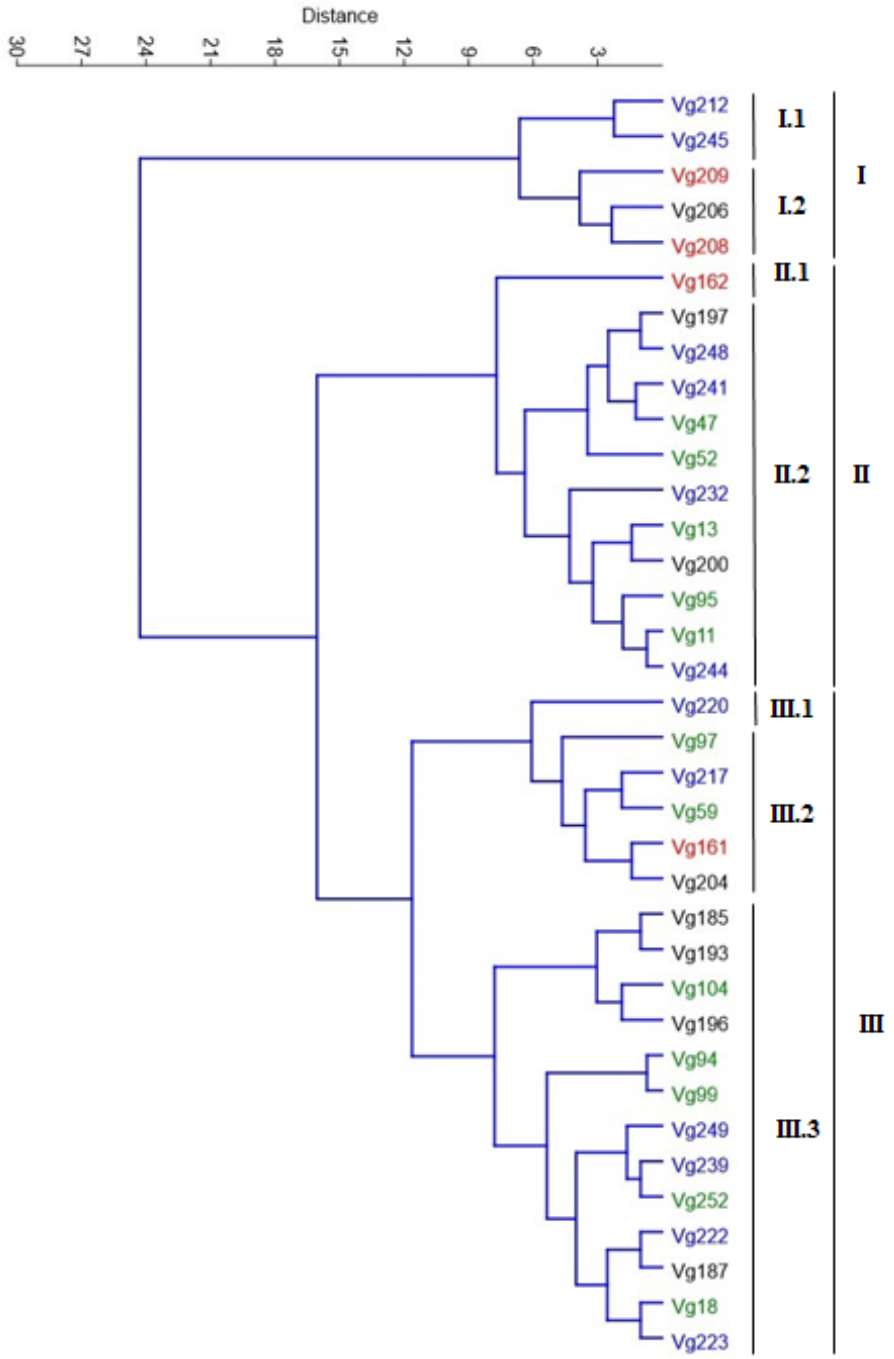

Figure 5 - Dendrogram of relationships among 36 cowpea landraces, by the Ward method, based on morphological traits. (Green – Portuguese landraces; Blue – Spanish landraces; Black – Italian landraces; Red – Greek landraces).

The principal component analysis (PCA) showed that the first three principal components explain 82.1 % of the total variation (PC1 = 63.7%; PC2 = 11.1% and PC3 = 7.3%) (Table 8). For the landraces separation, the major traits were: in the first component, the total seed weight (TSW) (0.89); in the second component, the pod length (PL) (0.65); and in the third component, the hilum colour (HC) (0.76) (Table 8).

Table 8 - Association of coefficients and vectors with the three axes of principal component analysis. (PL - Pod Length; NSP - Number of Seeds per Pod; SW - 100 Seeds Weight; GH - Growth Habit; TLS - Terminal Leaflet Shape; FC - Flower Colour; TSW - Total Seed Weight; SC - Seed Colour; HC - Hilum Colour; SS - Seed Shape).

	PC 1	**PC 2**	**PC3**
Eigenvalues	10.7	1.9	1.2
Percentage	63.7	11.1	7.3
Cumulative percentage	63.7	74.8	82.1
	PC1	**PC2**	**PC3**
PL	-0.16	0.65	0.14
NSP	0.04	0.26	-0.02
SW	-0.13	0.27	0.05
GH	0.24	-0.23	-0.19
TLS	-0.01	-0.33	0.58
FC	0.06	-0.07	-0.05
TSW	0.89	0.28	-0.10
SC	0.13	-0.12	-0.14
HC	0.26	0.06	0.76
SS	0.15	-0.42	-0.03

PCA and dendrogram are concordant (Figures 5 and 6). The landraces, are distributed by three main groups, coincident with the three dendrogram clusters, and it is possible to verify the distancing of Greek landrace Vg 162 in the group II and the Spanish Vg 220 in the group III. The projection of the 10 agronomic traits analysed in the plan defined by components 1 and 2 is shown in Figure 7. The trait number of seeds per pod is the main responsible for the individualization of the Greek landrace Vg 162, while for the Spanish landrace Vg 220 are the parameters total seed weight, and number of seeds per pod.

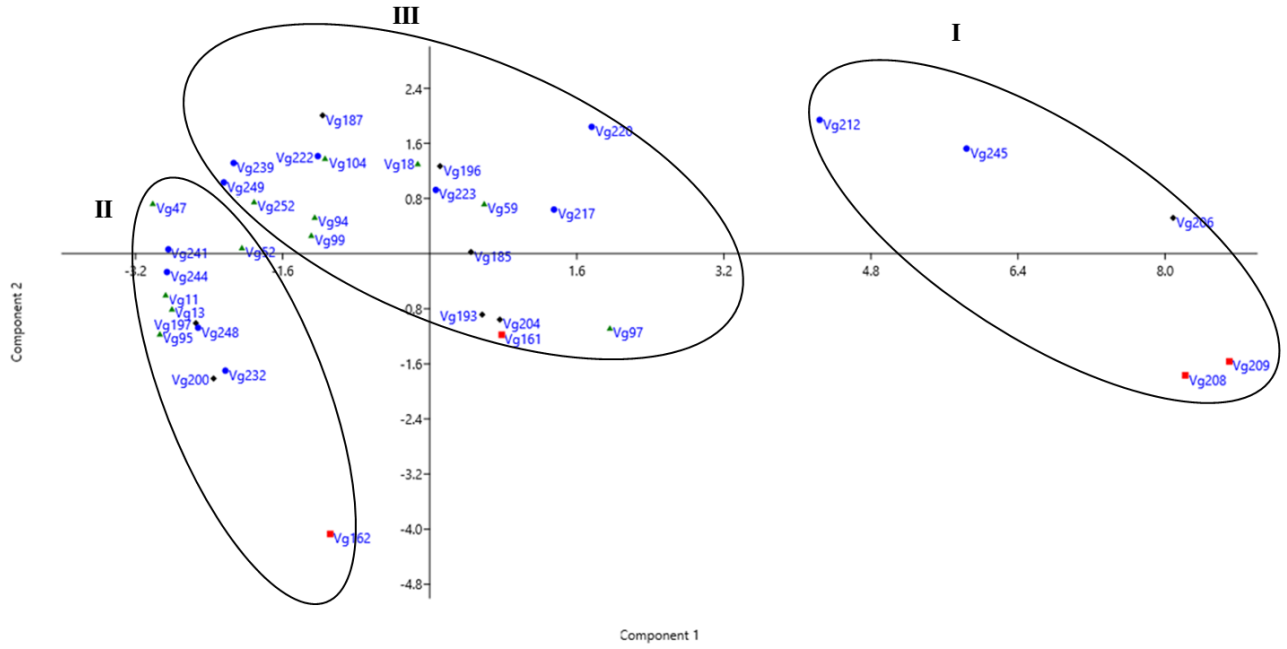

Figure 6 - Principal Components Analyses (PCA) of 36 cowpea landraces, based on 10 agronomic traits. (Green – Portuguese landraces; Blue – Spanish landraces; Black – Italian landraces; Red – Greek landraces).

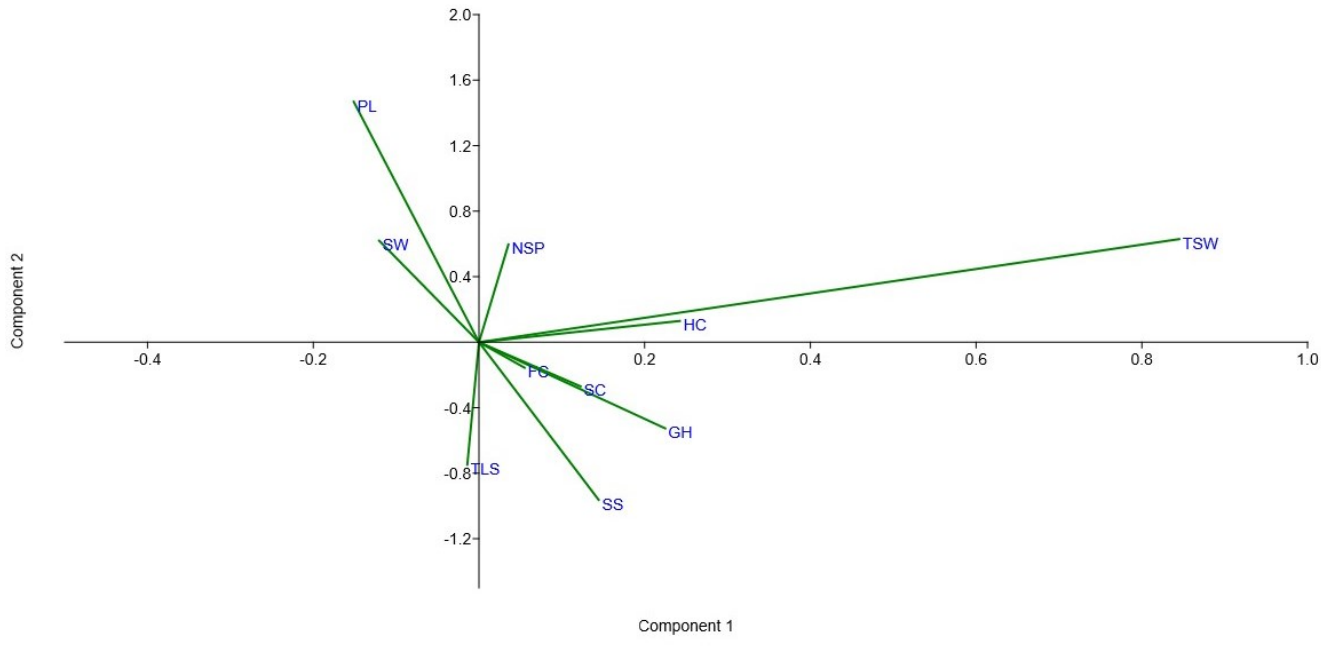

Figure 7 - Projection of the 10 morphological characteristics in axe 1 and 2. (PL - Pod Length; NSP - Number of Seeds per Pod; SW - 100 Seeds Weight; GH - Growth Habit; TLS - Terminal Leaflet Shape; FC - Flower Colour; TSW - Total Seed Weight; SC – Seed Colour; HC - Hilum Colour; SS - Seed Shape).

In order to help plant breeders to develop appropriate breeding strategies, to create the most adaptive and productive cultivars, knowledge of phenotypic variation and genotype relationships is essential. The study on landraces variation in morphological, phenological and agronomic traits is useful in the development of new cultivars with higher tolerance to biotic and abiotic stress factors, as well as with high yield potential.

This characterization allowed to verify the great diversity among Southern European cowpea landraces which may be useful in future breeding programs to obtain new varieties. Whereas, some of the morphological characteristics are influenced by the environment it would be important to have the results of one or two more growing seasons.

3.2. Chloroplast SSRs analysis of *Vigna unguiculata* and other *Vigna* species

A set of ten pairs of primers designed by Weising and Gardner (1999), Chung and Staub (2003) and Pan et al. (2014) were used to analyse the genetic diversity of 113 *Vigna* accessions, including mainly cultivated cowpea of unguiculata cultigroup. Eight (ccmp3, ccmp7, VgcpSSR1, VgcpSSR10, VgcpSSR12, VgcpSSR14 ccSSR4, cSSR7) out of the ten chloroplast microsatellite loci screened were polymorphic (Tables 9). The number of amplified alleles per primer pair ranged from one to five. Thus, the level of microsatellite polymorphism is low, when compared with other crops, such as common bean (*Phaseolus vulgaris*), where the number of alleles per loci ranged between 2 to 12 (Desiderio et al., 2013) and in rice (*Oryza sativa*) that raged from 2 to 9 (Herrera et al., 2008). One possible reason for this is that the materials used in the present study are mostly from the Iberian Peninsula and therefore had a relatively narrow genetic basis. Another possible reason for the low level of microsatellite polymorphism is the fact that a single domestication event is involved in the origin of this crop (Pasquet, 1999; Desalegne et al., 2016), unlike *P. vulgaris* (Singh et al., 1991) or rice (Second, 1985). Thus explaining the low genetic diversity in cowpea cultivated in relation to many other crops, especially legumes (Pasquet, 1993, 1999, Li et al., 2001, Asare et al., 2010). Thus the low genetic diversity of cowpea may be the result of this narrow genetic base.

The most frequent alleles were: 94 (100%) in ccmp10; 179 (100%) in ccSSR22; 79 (98.2%) in ccmp3; 313 (97.3%) in ccSSR7; 236 (96.5%) in VgcpSSR12; 146 (95.6%) in ccmp7; 262 (95.6%) in VgcpSSR1; 194 (94.7%) in VgcpSSR10; 256 (94.7%) in ccSSR4; 231 (92.9%) in VgcpSSR14. The genetic diversity varied from 0.000 to 0.135 and was

measured by the allele variation at the ten loci, with the minimum value in the polymorphic loci of 0.035 in ccmp3, and maximum value of 0.135 in VgcpSSR14 (Table 9). The number of effective alleles (*Ne*) and Shannon's information index (*I*) was also higher in the locus VgcpSSR14. The locus ccmp3 gave the lowest genetic diversity (*h*) (0.035) within the polymorphic loci detected in the accessions used in this study (Table 9).

Table 9 - Allele sizes (bp) and their frequencies, number of effective alleles (Ne), Shannon's information index (I), and genetic diversity (h) for the ten loci amplified in 113 Vigna accessions.

Locus	**Allele size (frequency)**	**Ne**	***I***	***h***
ccmp3	79 (0.982)	1.036	0.089	0.035
	84 (0.018)			
ccmp7	146 (0.956)	1.094	0.223	0.086
	147 (0.027)			
	148 (0.009)			
	149 (0.009)			
cmp10	94 (1.000)	1.000	0.000	0.000
VgcpSSR1	248 (0.009)	1.094	0.228	0.086
	249 (0.018)			
	259 (0.018)			
	262 (0.956)			
VgcpSSR10	166 (0.009)	1.114	0.278	0.103
	167 (0.018)			
	184 (0.018)			
	193(0.009)			
	194 (0.947)			
VgcpSSR12	236 (0.965)	1.074	0.190	0.069
	238 (0.018)			
	240 (0.009)			
	251 (0.009)			
VgcpSSR14	207 (0.009)	1.156	0.349	0.135
	216 (0.018)			
	223 (0.018)			
	231 (0.929)			
	233 (0.027)			
ccSSR4	256 (0.947)	1.114	0.274	0.102
	258 (0.009)			
	263 (0.027)			
	276 (0.009)			
	277 (0.009)			
ccSSR7	304 (0.009)	1.055	0.139	0.052
	312 (0.018)			
	313 (0.973)			
ccSSR22	179 (1.000)	1.000	0.000	0.000

Ten different haplotypes were obtained in this study, being the haplotype I the most frequent (90.3%), followed by haplotype II (2.7%). and the remaining haplotypes (III to X) unique (0.9%) (Table 10).

Table 10 - Chloroplast SSR haplotype verified for the 10 loci analysed and respective frequency.

	cpSSR loci										
Haplotype	**ccmp 3**	**ccmp 7**	**ccmp 10**	**VgcpSSR 1**	**VgcpSSR 10**	**VgcpSSR 12**	**VgcpSSR 14**	**ccSSR 4**	**ccSSR 7**	**ccSSR 22**	**Frequency**
I	79	146	94	262	194	236	231	256	313	179	90.3%
II	79	146	94	262	194	236	233	256	313	179	2.7%
III	79	146	94	262	194	236	231	258	313	179	0.9%
IV	79	146	94	262	193	236	231	256	313	179	0.9%
V	79	147	94	262	194	236	231	256	313	179	0.9%
VI	84	148	94	249	167	240	216	263	313	179	0.9%
VII	84	149	94	249	167	236	216	263	313	179	0.9%
VIII	79	146	94	259	184	238	223	277	312	179	0.9%
IX	79	147	94	259	184	238	223	276	312	179	0.9%
X	79	147	94	248	166	251	207	263	304	179	0.9%

In the Figure 8 is possible to verify the existence of ten different haplotypes. Of these haplotypes, eight were unique. The most frequent haplotype (I) includes *V. unguiculata* ssp. *unguiculata* cultigroup unguiculata (yellow), and cultigroup sesquipedalis (green), *V. unguiculata* ssp. *unguiculata* var. *spontanea* (blue) and *V. unguiculata* ssp. *tenuis* (black). The second most frequent haplotype (II) includes *V. unguiculata* ssp. *unguiculata* from Democratic Republic of Congo (yellow) and specimens of *V. unguiculata* ssp. *alba* (brown). The unique haplotypes (III-X) include *V. unguiculata* ssp. *pubescens* (red) (III-IV), *V. unguiculata* ssp. *tenuis* (black) (V), *V. mungo* (orange) (VI) *V. mungo sylvestris* (purple) (VII), *V. racemosa* (pink) (VIII-IX) and *V. radiata* (lilac) (X).

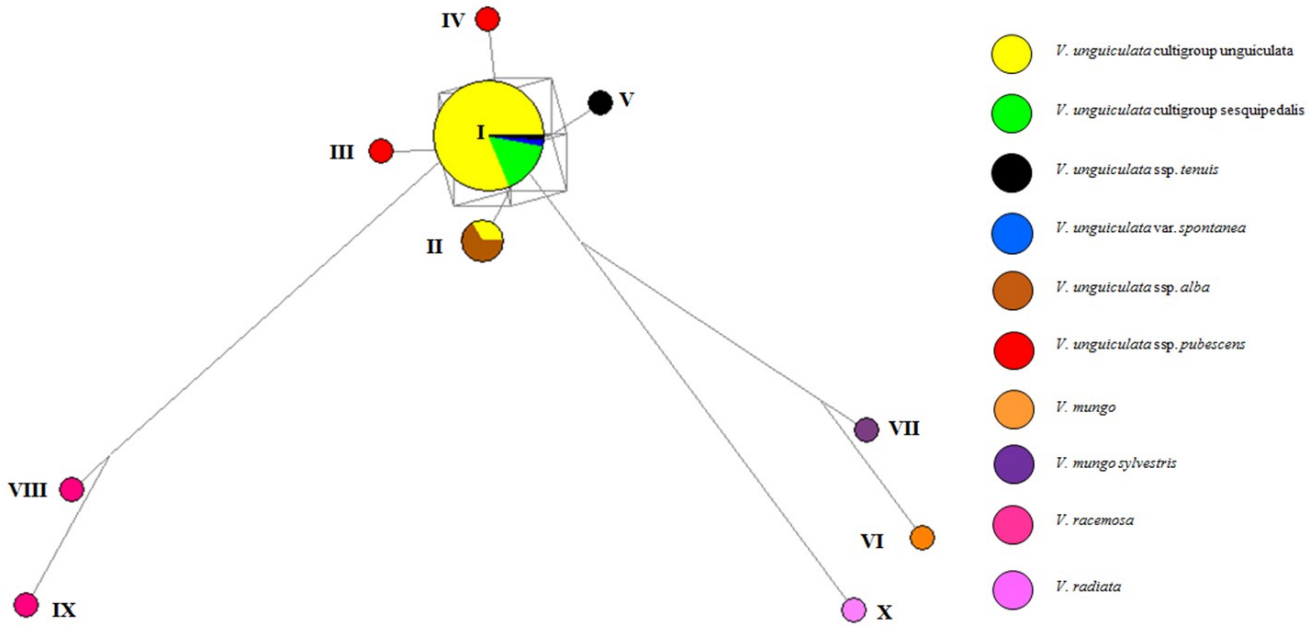

Figure 8 - Median-joining network of the haplotypes observed in 113 cowpea Vigna accessions (the area of the circle is proportional to haplotype frequency).

The clustering of the same data in a dendrogram (Figure 9) enabled to distinguish the 10 haplotypes, as in the Network projection (Figure 8). The 113 *Vigna* accessions were organized in three main clusters:

i) Cluster I, with all the cultivated *V. unguiculata* cultigroup unguiculata and *V. unguiculata* cultigroup sesquipedalis, the wild ssp. *unguiculata* var. *spontanea* and the accessions of *V. unguiculata* ssp. *tenuis from* Mozambique (Cluster I, haplotype I); the remaining sample of cultivated *V. unguiculata* ssp. *unguiculata* with origin in the Democratic Republic of Congo and the specimens of *V. unguiculata* ssp. *alba* (Cluster I, haplotype II). In the same cluster I also appear the two specimens of V. *unguiculata* ssp. *pubescens* (with different haplotype) and the specimen of V. *unguiculata* ssp. *tenuis* from Zambia. This high diversity within the V. *unguiculata* subspecies was also verified by Vijaykumar et al. (2009); these authors using nuclear ribosomal RNA internal transcriber spacer (ITS) verified that *Vigna unguiculata* accessions belonging to certain subspecies (namely ssp. *unguiculata* var. s*pontanea*, ssp. *tenuis*, and ssp. *alba*) were more close to accessions of other subspecies. The close clustering of accessions belonging to different subspecies has been attributed to

hybridization (or introgression) among them (Pasquet 1999; Coulibaly et al. 2002).

ii) Cluster II, with the two haplotypes of the species *V. mungo*, one wild and the other cultivated, the closest to *V. unguiculata*.

iii) Cluster III, with the two haplotypes of *V. racemosa.*

iv) Cluster IV formed by the outgroup species *V. radiata*.

It was further verified the ability of cpSSR markers in distinguishing the *Vigna* species of the two subgenus: the African subgenus *Vigna*, formed by *V. unguiculata* and its subspecies and the Asian subgenus *Ceratotropis* to which belong the other species of *Vigna* (Vijaykumar et al., 2009). This separation was also verified in studies of Fatokun et al. (1993) using RFLP analysis and Ajibade et al. (2000) using ISSR markers.

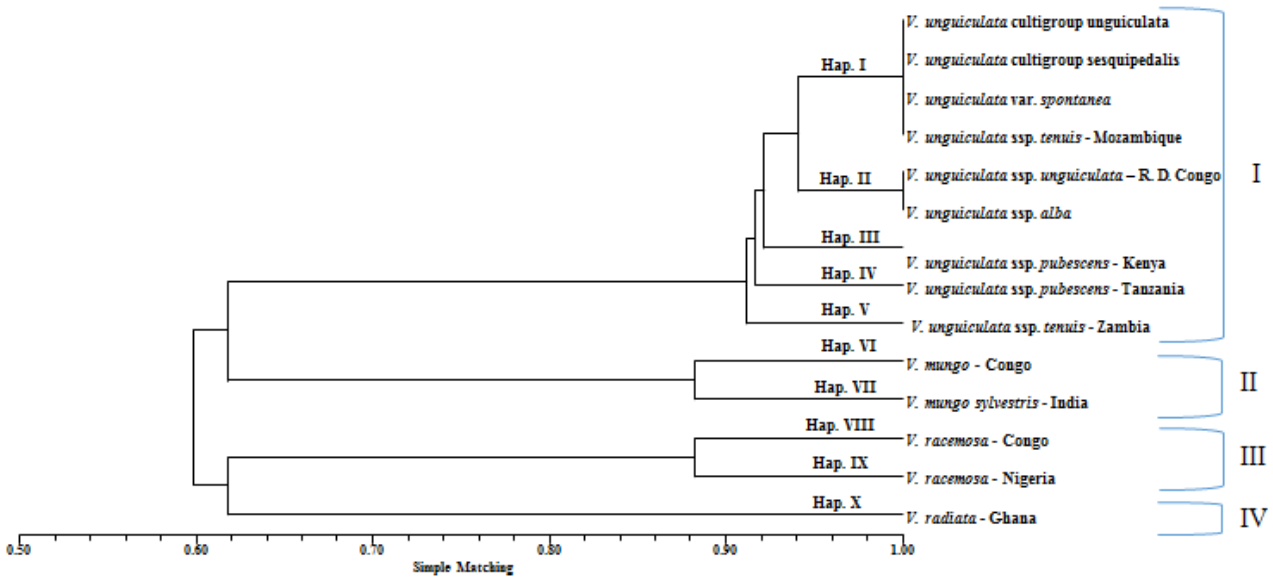

Figure 9 - Dendrogram of relationships between 113 Vigna accessions, by the UPGMA method, based on 10 cpSSRs markers.

In this study it was not possible to differentiate the cultigroups unguiculata and sesquipedalis. Moreover, these two cultigroups, shared haplotype with the wild var. s*pontanea*, which is referred to be the most likely progenitor of domesticated cowpea (Coulibaly et al., 2002 and Fang et al., 2007). In others studies of Fatokun et al. (1993) and Vijaykumar et al. (2012), it has already been verified that cultigroup sesquipedalis shows high phyletic relationship with cultigroup unguiculata and its wild relative var. *spontanea*.

It is known that both cowpea cultivated forms unguiculata and sesquipedalis are products of a post evolution of domestic *V. unguiculata* in different parts of the world (Fatokun et al., 1993). Whereas the African use of unguiculata cultigroup pulses remained unchanged over time, sesquipedalis cultigroup became established as a long-podded vegetable in Asia (Smartt, 1985). Selection practised for succulent and fleshy pod types among *V. unguiculata* introduced to Asia, especially in India, gave rise to the present-day yard-long bean (*V. unguiculata* cultigroup sesquipedalis) (Fatokun et al., 1993). The shared haplotype between these two forms of cultivated cowpea confirms the highly conserved nature of chloroplast genome with low mutation rate (Palmer, 1985; Vaillancourt and Weeden, 1992; Kapil et al., 2014).

The five different haplotypes now verified within the *V. unguiculata* subspecies represents a considerable evolution in the chloroplastidial genome. In the Figure 8, we can observe that the most ancestral haplotype should be the one of var. *spontanea* and the remaining subspecies of *V. unguiculata* and species of *Vigna* should have diverged from it. It is also possible to confirm, as expected, that the species *V. radiata*, *V. mungo* and *V. racemosa* are more distant from the ancestor than the different subspecies of *V. unguiculata*.

When we analyse the subspecies of *V. unguiculata*, we verified that the accession of *V. unguiculata* cultigroup unguiculata from the Democratic Republic of Congo is closer to the ssp. *alba* than to its ancestor (var. *spontanea*). The close geographic origin of the two accessions of ssp. *alba*, one also from the Democratic Republic of Congo and the other from Angola, can explain the same haplotype of these accessions.

Accessions of the ssp. *tenuis* showed divergence and did not cluster together, because within this subspecies we had accessions close to the ancestor (var. *spontanea*) with the same haplotype, but also accessions with a different haplotype.

In the case of ssp. *pubescens* two different haplotypes were detected, such as for ssp. *tenuis* and neither of them identical to var. *spontanea*. One explanation for these two ssp. *pubescens* haplotypes can be that cowpea, like mungbean (*V. radiata*) and bambara groundnut (*V. subterranea*), is highly self-pollinating (Fatokun et al., 1993; Otwe et al., 2017) and so, the differences observed within accessions can be attributed to a mixture of seeds or variations caused by mutations or reduced cross pollination.

Understanding genetic variation is very important for germplasm management and developing collections in order to provide raw material to breeders and farmers to improve

high productivity through plant breeding. In our study the low level of polymorphism detected within the cultivated cowpea is in agreement with previous studies reported by several researchers and may be the result of a single domestication event in this crop or to its inherent nature of self-pollination mechanism (Li et al., 2001; Tosti and Negri, 2002; Diouf and Hilu, 2005; Badiane et al., 2012).

4. Conclusion

In this study, we proceed to the morphological, agronomic and molecular characterization of several *Vigna* species and *Vigna unguiculata* subspecies, with the aim to characterize landraces from Southern Europe and evaluate the diversity and genetic relationships between worldwide accessions, using cpSSRs markers.

Since cowpea is a legume resistant to different biotic and abiotic factors and contains important nutritional factors, its characterization, both morphologically and molecularly, is extremely important for a better knowledge about its taxonomy, phylogenetic relationships, but also for breeding programs.

Morphological and agronomic characterization should be the first step in the characterization of a culture, but it has certain disadvantages, since the characteristics observed do not reflect exclusively the genotype, but reflect the environment and the interaction genotype and environment effects. Therefore, even after the morphological characterization an analysis at the molecular level is always necessary. In this study the molecular markers used were the cpSSRs, because the chloroplast genome is quite conserved, which allows us to have a view of the evolution of the genome of this species, once we analysed cultivated and wild forms.

The morphological and agronomic characterization of landraces from Southern Europe, revealed that the most frequent quantitative characteristics were the growth habit erect, terminal leaflet type sub-hastate shape, white flowers, seeds of cream colour, with kidney shape and black hilum. These results may demonstrate that people in Southern Europe have a preference for these characteristics at the seed level, but at the same time we can say that this choice may reflect the fact that these characteristics are associated with high grain yield and grain quality. It was also verified that within the quantitative characteristics the character with higher coefficient of variation was total seed weight and the one with lower was pod length. The characteristic 100 seeds weight presented the highest heritability value, although high heritability alone is not enough to make efficient selection in the advanced generations unless accompanied by substantial amount of genetic advance. There was no relationship between the clustering of the 36 Southern Europe cowpea landraces and their geographical origin, although a few characteristics were only observed in grains of the same country. This characterization was important for cowpea future breeding

programs in Southern European countries providing agro-morphological information of its germplasm, with focus on Iberian Peninsula's.

In relation to the characterization using the cpSSRs, it was verified that the level of polymorphisms is low within the cultivated cowpeas, when compared to other crops. This is possible due to the fact that the material in this study is mostly from the Iberian Peninsula and therefore had a relatively narrow genetic basis, or because of the fact that a single domestication event is involved in the origin of this crop. Ten different haplotypes were found, being the haplotype I the most frequent and, putatively, the ancestral haplotype, since it includes not only the wild var. *spontanea* (the progenitor of the cowpea), but also the cultivated ssp. *unguiculata* and ssp. *tenuis*. It was found that different accessions of the ssp. *tenuis* and ssp. *pubescens* present different haplotypes. The other species of *Vigna* seem to diverge from the subspecies of *unguiculata*, this evolution may have happened gradually, since *V. racemosa* presents two different haplotypes that derive from the ancestor. In the case of *V. mungo*, are in concordance that the wild form *sylvestris* is more ancestral than the cultivated form *V. mungo*. However, these results need to be confirmed, since the taxon's *V. racemosa* and *V. unguiculata* ssp. *tenuis* and ssp. *pubescens* presented two different haplotypes, which may indicate a divergence during their evolution, or the existence of taxonomic errors.

More molecular studies would be necessary, namely using other primers, a larger number of markers and sampling of cultivated cowpea in different locations to better understand the genetic basis of this crop and also analyse wild relative including other species of *Vigna* and subspecies of *V. unguiculata* to comprehend their phylogenetic relationship.

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6. Supplementary material

Table S1- Origin of the Iberian Peninsula cultivated *V. unguiculata* spp. *unguiculata*, cultigroups unguiculata and sesquipedalis accessions studied.

Accession number	Bank code[#]	Cultigroup	Locality	Latitude	Longitude	Altitude (m)	Common name
Vg160	Faial	sesquipedalis	Açores, Faial	-	-	-	Feijão a metro
Vg11	Vg11	unguiculata	Torre de Moncorvo	4127506N	705272W	-	Feijão frade
Vg13	Vg13	unguiculata	Alijó	4119020N	724310W	517	Feijão frade
Vg47	Vg47	unguiculata	Almeida	4035595N	653595W	794	Feijão frade
Vg97	CP5648	unguiculata	Abrantes	3927530N	802448W	45	Feijão frade
Vg99	CP5651	unguiculata	Ponte de Sor	3916223N	800449W	119	Feijão frade
Vg69	Vg69	unguiculata	Bragança	4140197N	645439W	859	Feijão frade
Vg158	CPS-8	sesquipedalis	Bragança	-	-	-	Feijão a metro
Vg64	Vg64	unguiculata	Celorico da Beira	4037343N	724296W	519	Feijão frade
Vg62	Vg62	unguiculata	Covilhã	4017521N	722016W	511	Feijão frade
Vg88	CP4924	unguiculata	Évora	3833529N	756286W	258	Feijão frade
Vg86	CP4847	unguiculata	Ferreira do Alentejo	-	-	-	Feijão frade
Vg48	Vg48	unguiculata	F.Castelo Rodrigo	4048041N	656594W	663	Feijão frade
Vg59	Vg59	unguiculata	Fundão	4014572N	717227W	507	Feijão frade
Vg91	CP5128	unguiculata	Lardosa	3959114N	726398W	402	Feijão frade
Vg56	Vg56	unguiculata	Macedo de Cavaleiros	4144383N	738575W	673	Feijão frade
Vg51	Vg51	unguiculata	Meda	4050585N	715163W	580	Feijão frade
Vg15	Vg15	unguiculata	Miranda do Douro	4126220N	623310W	729	Feijão frade
Vg18	Vg18	unguiculata	Mirandela	4138030N	712087W	370	Feijão frade
Vg72	Vg72	unguiculata	Mogadouro	4116571N	635060W	726	Feijão frade

[#] Vg, University of Trás-os-Montes and Alto Douro, Vila Real, Portugal; CP, National Institute for Agricultural and Veterinary Research (INIAV), Elvas, Portugal; BGE, National Plant Genetic Resources Centre-National Institute for Agricultural and Food Technology Research (CRF-INIA), Alcalá de Henares, Spain; NC, Centro de Investigación y Tecnología Agroalimentaria. Banco de Germoplasma de Hortícolas, Zaragoza, Spain.

Table S1 - Origin of the Iberian Peninsula cultivated *V. unguiculata* spp. *unguiculata*, cultigroups unguiculata and sesquipedalis accessions studied (continued).

Accession number	Bank code[#]	Cultigroup	Locality	Latitude	Longitude	Altitude (m)	Common name
Vg95	CP5556	unguiculata	Mértola	3747158N	743329W	160	Feijão frade
Vg58	Vg58	unguiculata	Penamacor	4017070N	707200W	607	Feijão frade
Vg49	Vg49	unguiculata	Pinhel	4047549N	703282W	573	Feijão frade
Vg87	CP4906	unguiculata	Ansião	4000288N	827043W	198	Feijão frade
Vg96	CP5647	unguiculata	Gavi	-	-	-	
Vg12	Vg12	unguiculata	Bragança	4156250N	637000W	719	Feijão frade
Vg60	Vg60	unguiculata	Sabugal	4022009N	715325W	514	Feijão frade
Vg94	CP5553	unguiculata	Sertã	3948029N	806035W	226	Feijão frade
Vg104	CP5554	unguiculata	Sousel	-	-	-	Feijão frade
Vg52	Vg52	unguiculata	Trancoso	4048451N	723260W	770	Feijão frade
Vg54	Vg54	unguiculata	Valpaços	4144383N	738575W	673	Feijão frade
Vg101	CP5645	unguiculata	Vila Nova de Ourém	-	-	-	Feijão frade
Vg85	CP5263	sesquipedalis	-	-	-	-	Feijão frade
Vg252	Vg252	unguiculata	Baião	-	-	-	Feijão frade
Vg245	BGE028976	unguiculata	Albacete, Yeste	382407N	022610W	1100	Ciriguello
Vg230	BGE043764	unguiculata	Alicante, Lorcha	385043N	001836W	268	Careto
Vg251	BGE031003	unguiculata	Avila, Candeleda	400913N	051416W	432	Carilla
Vg222	BGE024703	unguiculata	Baleares, Palma de Mallorca	393425N	023910E	19	Fesol
Vg227	BGE040818	sesquipedalis	Cadiz, Jerez de la Frontera	363851N	055527W	20	Habichuela de verdeo
Vg228	BGE040819	sesquipedalis	Cadiz, Zahara	365030N	052430W	324	Chicharo
Vg240	BGE039238	sesquipedalis	Cordoba, Baena	373652N	041940W	462	Judia antigua

[#] Vg, University of Trás-os-Montes and Alto Douro, Vila Real, Portugal; CP, National Institute for Agricultural and Veterinary Research (INIAV), Elvas, Portugal; BGE, National Plant Genetic Resources Centre-National Institute for Agricultural and Food Technology Research (CRF-INIA), Alcalá de Henares, Spain; NC, Centro de Investigación y Tecnología Agroalimentaria. Banco de Germoplasma de Hortícolas, Zaragoza, Spain.

Table S1 - Origin of the Iberian Peninsula cultivated *V. unguiculata* spp. *unguiculata*, cultigroups unguiculata and sesquipedalis accessions studied (continued).

Accession number	Bank code[#]	Cultigroup	Locality	Latitude	Longitude	Altitude (m)	Common name
Vg249	BGE039237	unguiculata	Cordoba, Baena	374015N	041412W	336	Higuelo
Vg236	BGE035391	unguiculata	Badajoz, Garlitos	385301N	050247W	554	Carilla
Vg244	BGE035390	unguiculata	Badajoz, Oliva de la Frontera	381645N	0065502W	380	Frailiño careto
Vg223	BGE025201	unguiculata	Caceres, Villanueva de la Vera	4006--N	00524--W	413	Carilla
Vg224	BGE025213	unguiculata	Caceres, Arroyomolinos de laVera	400315N	0055115W	617	Minine
Vg217	BGE019751	unguiculata	Gerona, La Bisbal d'Emporda	415740N	0030222E	39	Frijol d'hiver
Vg220	BGE022147	unguiculata	Granada, Portugos	365634N	0031835W	1302	Friguelo
Vg247	BGE040000	sesquipedalis	Granada, Cortes de Baza	373732N	0024704W	679	Habilla
Vg239	BGE036461	unguiculata	Huelva, Villanueva de los Castillejos	373007N	0071616W	231	Carilla
Vg191	NC105325	unguiculata	Huesca, Fraga	413119N	0002056W	125	Ojo de perdiz
Vg192	NC105327	sesquipedalis	Huesca, Ballobar	413719N	0001127W	162	Metrera
Vg229	BGE041751	sesquipedalis	Jaen, Albanchez de Magina	374729N	0032755W	859	Habicholon
Vg241	BGE039236	unguiculata	Jaen, Castillo de Locubin	373149N	0035637W	688	Jiguelo
Vg237	BGE038476	sesquipedalis	Malaga, Alhaurin el Grande	-	-	-	Habichuela larga
Vg243	BGE038474	unguiculata	Malaga, Genalguacil	-	-	-	Chicharo
Vg226	BGE027108	sesquipedalis	Murcia, Mula	3809--N	00133--W	403	Bisuelo
Vg84	Vi4	sesquipedalis	Murcia,	-	-	-	
Vg231	BGE044375	unguiculata	Orense, Cenlle	421955N	0080130W	122	Xudia
Vg212	BGE002195	unguiculata	Orense, Lobios	415435N	0080408W	330	Carilla
Vg232	BGE047731	unguiculata	Pontevedra, Arbo	420553N	0082057W	97	Cajabicho
Vg250	BGE037805	sesquipedalis	Sevilla, Casariche	371748N	0044528W	294	Habichuela larga

[#] Vg, University of Trás-os-Montes and Alto Douro, Vila Real, Portugal; CP, National Institute for Agricultural and Veterinary Research (INIAV), Elvas, Portugal; BGE, National Plant Genetic Resources Centre-National Institute for Agricultural and Food Technology Research (CRF-INIA), Alcalá de Henares, Spain; NC, Centro de Investigación y Tecnología Agroalimentaria. Banco de Germoplasma de Hortícolas, Zaragoza, Spain.

Table S1 - Origin of the Iberian Peninsula cultivated *V. unguiculata* spp. *unguiculata*, cultigroups unguiculata and sesquipedalis accessions studied (continued).

Accession number	Bank code[#]	Cultigroup	Locality	Latitude	Longitude	Altitude (m)	Common name
Vg221	BGE024406	sesquipedalis	Tarragona, Riudecanyes	410752N	0005743E	195	Judia
Vg235	BGE036462	unguiculata	Valencia, Carcaixent	390726N	0002645W	20	Judia careta
Vg248	BGE040426	unguiculata	Zamora, Asturianos	420311N	0062916W	970	Carilla
Vg190	NC105329	sesquipedalis	Zaragoza, Barrio Oliver	413860N	0005260W	208	Judia larga

[#] Vg, University of Trás-os-Montes and Alto Douro, Vila Real, Portugal; CP, National Institute for Agricultural and Veterinary Research (INIAV), Elvas, Portugal; BGE, National Plant Genetic Resources Centre-National Institute for Agricultural and Food Technology Research (CRF-INIA), Alcalá de Henares, Spain; NC, Centro de Investigación y Tecnología Agroalimentaria. Banco de Germoplasma de Hortícolas, Zaragoza, Spain.

Table S2 - Other countries cultivated *V. unguiculata* spp. *unguiculata*, cultigroups unguiculata and sesquipedalis accessions studied.

Accession number	Bank code[#]	Cultigroup	Country	Status	Common name
Vg138	NI 206	unguiculata	Angola	Landrace	-
Vg152	-	unguiculata	Brazil	Breeding	Miudo Preto Aparecido
Vg154	-	unguiculata	Brazil	Commercial cultivar	Nordeste
Vg155	-	unguiculata	Brazil	Commercial cultivar	Miudo Mamoninha
Vg156	-	unguiculata	Brazil	Commercial cultivar	Baio Coofam
Vg28	A4 E 007	unguiculata	Bulgaria	-	-
Vg29	A4 E 008	unguiculata	Bulgaria	-	-
Vg32	Vg 87210026	unguiculata	Bulgaria	-	-
Vg34	Vg 95210023	unguiculata	Bulgaria	-	-
Vg125	VIG 10	unguiculata	China	Landrace	-
Vg144	NI 1183	unguiculata	China	Landrace	-
Vg151	NI 262	sesquipedalis	China	Landrace	-
Vg140	NI 22	unguiculata	D.R. Congo	Landrace	-
Vg137	VIG 206	unguiculata	Cuba	Landrace	-
Vg117	VIG 66	unguiculata	Egypt	Landrace	-
Vg116	VIG 90	unguiculata	Egypt	-	-
Vg118	VIG 71	unguiculata	Ghana	-	-
Vg161	AUA1	unguiculata	Greece	-	-
Vg162	AUA2	unguiculata	Greece	-	-
Vg208	MG 106823	unguiculata	Greece	Landrace	Mavromatica
Vg209	MG 107571	unguiculata	Greece	Landrace	Lianofasula
Vg146	NI 778	unguiculata	India	Landrace	-
Vg147	NI 784	unguiculata	India	Landrace	-
Vg127	VIG 1650	unguiculata	Iran	Landrace	-
Vg130	VIG 100	unguiculata	Iraq	Landrace	-
Vg187	5426	unguiculata	Italy	-	-
Vg193	MG 115107	unguiculata	Italy	-	-
Vg204	MG 113779	unguiculata	Italy	Landrace	Fagiolini pinti baresi
Vg206	MG 112248	unguiculata	Italy	-	-
Vg132	VIG 87	unguiculata	Libya	-	-
Vg142	NI 1139	unguiculata	Madagascar	Landrace	-
Vg159	-	unguiculata	Nigéria	Landrace	-
Vg120	VIG 49	unguiculata	Senegal	-	-
Vg123	VIG 51	unguiculata	Zambia	-	-

[#] Vg, University of Trás-os-Montes and Alto Douro, Vila Real, Portugal; AUA, University of Athens, Athens, Greece; MG, Institute of Biosciences and Bioresources (IBBR), Italian National Research Council (CNR), Bari, Italy; NI, Botanic Garden Meise, Belgium; VIG, Leibniz Institute of Plant Genetics and Crop Plant Research (IPK) Gatersleben, Germany.

Table S3 - Origin of the accessions of wild *V. unguiculata* ssp *unguiculata* var. *spontanea,* other *V. unguiculata* subspecies and other *Vigna* species studied.

Accession number	Bank code[#]	Species	Country	Locality	Status
Vg259	NI 1656	*alba*	Angola	Between Maria Teresa and Culomboluca	Wild
Vg264	NI 1754	*alba*	D-R. Congo	Diosso - Kayes rd.	Wild
Vg260	NI 989	*pubescens*	Kenya	Kilifi distr., Whispering Palms Hotel	Wild
Vg262	NI 1862	*pubescens*	Tanzania	Korogwe	Wild
Vg257	NI 1655	*spontanea*	Madagascar	Diégo Suarez, Antsakoafe	Wild
Vg254	NI 963	*spontanea*	Senegal	Casamance, Cap Shirring	Wild
Vg256	NI 1808	*tenuis*	Mozambique	Inhaca Island	Wild
Vg263	NI 1664	*tenuis*	Zambia	Luanshya - Mpongwe rd.	Wild
Vg175	NI 207	*mungo*	D.R. Congo	Kasaï, INEAC Gandajika	Landrace
Vg168	NI 635	*mungo*	India	Maharashtra, Khandala	Wild
Vg170	NI 239	*racemosa*	D.R.Congo	Kasaï, INEAC Gandajika	Wild
Vg179	NI 977	*racemosa*	Nigéria	Wuga - Muresti rd.	Wild
Vg184	NI 159	*radiata*	Ghana	Accra	Landrace

[#] NI, Botanic Garden Meise, Belgium.

Table S4 – Alleles size at ten different cpSSRs loci of 113 accessions analysed.

Genotype	CpSSR loci									
	ccmp3	**ccmp7**	**ccmp10**	**VgcpSSR1**	**VgcpSSR10**	**VgcpSSR12**	**VgcpSSR14**	**ccSSR4**	**ccSSR7**	**ccSSR22**
Vg160	79	146	94	262	194	236	231	256	313	179
Vg11	79	146	94	262	194	236	231	256	313	179
Vg13	79	146	94	262	194	236	231	256	313	179
Vg47	79	146	94	262	194	236	231	256	313	179
Vg97	79	146	94	262	194	236	231	256	313	179
Vg99	79	146	94	262	194	236	231	256	313	179
Vg69	79	146	94	262	194	236	231	256	313	179
Vg158	79	146	94	262	194	236	231	256	313	179
Vg64	79	146	94	262	194	236	231	256	313	179
Vg62	79	146	94	262	194	236	231	256	313	179
Vg88	79	146	94	262	194	236	231	256	313	179
Vg86	79	146	94	262	194	236	231	256	313	179
Vg48	79	146	94	262	194	236	231	256	313	179
Vg59	79	146	94	262	194	236	231	256	313	179
Vg91	79	146	94	262	194	236	231	256	313	179
Vg56	79	146	94	262	194	236	231	256	313	179
Vg51	79	146	94	262	194	236	231	256	313	179
Vg15	79	146	94	262	194	236	231	256	313	179
Vg18	79	146	94	262	194	236	231	256	313	179
Vg72	79	146	94	262	194	236	231	256	313	179
Vg95	79	146	94	262	194	236	231	256	313	179
Vg58	79	146	94	262	194	236	231	256	313	179
Vg49	79	146	94	262	194	236	231	256	313	179
Vg87	79	146	94	262	194	236	231	256	313	179
Vg96	79	146	94	262	194	236	231	256	313	179
Vg12	79	146	94	262	194	236	231	256	313	179
Vg60	79	146	94	262	194	236	231	256	313	179
Vg94	79	146	94	262	194	236	231	256	313	179
Vg104	79	146	94	262	194	236	231	256	313	179
Vg52	79	146	94	262	194	236	231	256	313	179
Vg54	79	146	94	262	194	236	231	256	313	179
Vg101	79	146	94	262	194	236	231	256	313	179
Vg85	79	146	94	262	194	236	231	256	313	179
Vg252	79	146	94	262	194	236	231	256	313	179
Vg245	79	146	94	262	194	236	231	256	313	179
Vg230	79	146	94	262	194	236	231	256	313	179
Vg251	79	146	94	262	194	236	231	256	313	179

Table S4 – Alleles size at ten different cpSSRs loci of 113 accessions analysed (continued).

Genotype	CpSSR loci									
	ccmp3		**ccmp3**		**ccmp3**		**ccmp3**		**ccmp3**	
Vg 222	79	146	94	262	194	236	231	256	313	179
Vg227	79	146	94	262	194	236	231	256	313	179
Vg228	79	146	94	262	194	236	231	256	313	179
Vg240	79	146	94	262	194	236	231	256	313	179
Vg249	79	146	94	262	194	236	231	256	313	179
Vg236	79	146	94	262	194	236	231	256	313	179
Vg244	79	146	94	262	194	236	231	256	313	179
Vg223	79	146	94	262	194	236	231	256	313	179
Vg224	79	146	94	262	194	236	231	256	313	179
Vg217	79	146	94	262	194	236	231	256	313	179
Vg 220	79	146	94	262	194	236	231	256	313	179
Vg247	79	146	94	262	194	236	231	256	313	179
Vg239	79	146	94	262	194	236	231	256	313	179
Vg191	79	146	94	262	194	236	231	256	313	179
Vg192	79	146	94	262	194	236	231	256	313	179
Vg229	79	146	94	262	194	236	231	256	313	179
Vg241	79	146	94	262	194	236	231	256	313	179
Vg237	79	146	94	262	194	236	231	256	313	179
Vg243	79	146	94	262	194	236	231	256	313	179
Vg226	79	146	94	262	194	236	231	256	313	179
Vg84	79	146	94	262	194	236	231	256	313	179
Vg231	79	146	94	262	194	236	231	256	313	179
Vg212	79	146	94	262	194	236	231	256	313	179
Vg232	79	146	94	262	194	236	231	256	313	179
Vg250	79	146	94	262	194	236	231	256	313	179
Vg 221	79	146	94	262	194	236	231	256	313	179
Vg235	79	146	94	262	194	236	231	256	313	179
Vg248	79	146	94	262	194	236	231	256	313	179
Vg190	79	146	94	262	194	236	231	256	313	179
Vg138	79	146	94	262	194	236	231	256	313	179
Vg152	79	146	94	262	194	236	231	256	313	179
Vg154	79	146	94	262	194	236	231	256	313	179
Vg155	79	146	94	262	194	236	231	256	313	179
Vg156	79	146	94	262	194	236	231	256	313	179
Vg28	79	146	94	262	194	236	231	256	313	179
Vg29	79	146	94	262	194	236	231	256	313	179
Vg32	79	146	94	262	194	236	231	256	313	179

Table S4 – Alleles size at ten different cpSSRs loci of 113 accessions analyse

Genotype	CpSSR loci									
	ccmp7	**ccSSR4**	**ccmp3**	**VgcpSSR10**	**ccSSR22**	**ccmp10**	**VgcpSSR1**	**ccSSR7**	**VgcpSSR12**	**VgcpSSR14**
Vg34	146	256	79	194	179	94	262	313	236	231
Vg125	146	256	79	194	179	94	262	313	236	231
Vg144	146	256	79	194	179	94	262	313	236	231
Vg151	146	256	79	194	179	94	262	313	236	231
Vg140	146	256	79	194	179	94	262	313	236	233
Vg137	146	256	79	194	179	94	262	313	236	231
Vg117	146	256	79	194	179	94	262	313	236	231
Vg116	146	256	79	194	179	94	262	313	236	231
Vg118	146	256	79	194	179	94	262	313	236	231
Vg161	146	256	79	194	179	94	262	313	236	231
Vg162	146	256	79	194	179	94	262	313	236	231
Vg208	146	256	79	194	179	94	262	313	236	231
Vg209	146	256	79	194	179	94	262	313	236	231
Vg146	146	256	79	194	179	94	262	313	236	231
Vg147	146	256	79	194	179	94	262	313	236	231
Vg127	146	256	79	194	179	94	262	313	236	231
Vg130	146	256	79	194	179	94	262	313	236	231
Vg187	146	256	79	194	179	94	262	313	236	231
Vg193	146	256	79	194	179	94	262	313	236	231
Vg204	146	256	79	194	179	94	262	313	236	231
Vg206	146	256	79	194	179	94	262	313	236	231
Vg132	146	256	79	194	179	94	262	313	236	231
Vg142	146	256	79	194	179	94	262	313	236	231
Vg159	146	256	79	194	179	94	262	313	236	231
Vg120	146	256	79	194	179	94	262	313	236	231
Vg123	146	256	79	194	179	94	262	313	236	231
Vg259	146	256	79	194	179	94	262	313	236	233
Vg 264	146	256	79	194	179	94	262	313	236	233
Vg260	146	258	79	194	179	94	262	313	236	231
Vg262	146	256	79	193	179	94	262	313	236	231
Vg257	146	256	79	194	179	94	262	313	236	231
Vg254	146	256	79	194	179	94	262	313	236	231
Vg256	146	256	79	194	179	94	262	313	236	231
Vg263	147	256	79	194	179	94	262	313	236	231
Vg175	148	263	84	167	179	94	249	313	240	216
Vg168	149	263	84	167	179	94	249	313	236	216
Vg170	146	277	79	184	179	94	259	312	238	223
Vg179	147	276	79	184	179	94	259	312	238	223
Vg184	147	263	79	166	179	94	248	304	251	207